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EXPERIMENTAL RAT CARIES

I. PRODUCTION OF RAT CARIES IN THE PRESENCE OF ALL KNOWN NUTRITIONAL ESSENTIALS AND IN THE ABSENCE OF COARSE FOOD PARTICLES AND THE IMPACT OF MASTICATION

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SIX FIGURES

(Received for publication March 13, 1948)

Considerable evidence, reviewed elsewhere (Hodge and Sognaes, '46), indicates that the conventional type of experimental rat caries is initiated by mechanical injury to the teeth caused by the masticatory impaction of coarse food particles. Indeed, 1 author (Rosebury, '39) included all work on rat caries when he concluded that "the appearance of fissure caries among a group of animals, in whatever degree, is definite evidence that such a primary cause was present," and went on to formulate the concept that "dental caries in man is caused primarily by such food," i.e., by the impaction of coarse food particles.

Recent experimental and clinical studies (Sognaes, '47, '48a, b) were not compatible with such generalization of an oral environmental mechanism. From further experiments with the rat, additional evidence can now be presented to show (1) that rat caries can be produced by a ration which, besides being adequate in known nutritional essentials, is free from coarse food particles; (2) that the carious lesions will develop

¹The term "rat caries" refers here to the problem of caries production in the ordinary laboratory rat (*Mus Norvegicus*), and does not include the lesions produced in the newer experimental rodents (cotton rats, Syrian hamsters).

even if the rat molars are relieved from the impact of mastication; and (3) that the lesions are grossly and histologically comparable to human caries.

EXPERIMENTAL

1. *The properties of the caries-conducive purified ration*

The nutritional and physical composition of the purified ration is shown in tables 1 and 2, respectively. Nutritionally, this ration is essentially the same as that which, with the addition of ascorbic acid, has been found to produce good growth and health in the rhesus monkey (Waisman et al., '43)

TABLE 1
Composition of the caries-conducive purified ration

COMPONENT	AMOUNT	COMPONENT	AMOUNT
Sucrose, granulated (%)	67	<i>Vitamins</i>	
Casein (%)	24	Thiamine (p.p.m.)	3.5
Corn oil (%)	5	Riboflavin (p.p.m.)	3.5
Salt mixture ¹ (%)	4	Nicotinic acid (p.p.m.)	25.0
Calcium (%)	0.53	p-Aminobenzoic acid (p.p.m.)	200
Phosphorus (%)	0.55	Pantothenic acid (p.p.m.)	20
Magnesium (%)	0.033	Pyridoxine (p.p.m.)	3.5
Iron (p.p.m.)	150	Inositol (p.p.m.)	1000
Manganese (p.p.m.)	50	Choline (p.p.m.)	1000
Copper (p.p.m.)	15	Vitamin A (U.S.P. Units/gm.)	2000
Cobalt (p.p.m.)	0.65	Vitamin D (U.S.P. Units/gm.)	1.0
Potassium (%)	0.45		
Sodium (%)	0.44	Liver concentrate (%)	1
Chlorine (%)	0.76		
Iodine (p.p.m.)	20		
Zinc (p.p.m.)	30		
Sulfur (p.p.m.)	480		
Fluorine (p.p.m.) ²	2.5		

¹ Modification of Phillips and Hart's salt mixture (J. Biol. Chem., 109: 657, 1935) by Shaw, J. H. (J. Dental Res., 26: 47-51, 1947).

² This trace element is not added for general nutritional purposes but is due to minor impurities of the carbohydrate fraction and the salt mixture (Shaw et al., Proc. Soc. Exp. Biol. Med., 52: 89-92, 1945, and personal communication).

In the rodents, the identical purified ration has, in addition, been found to promote good reproduction, which is particularly true of the rat, with which species this and the preceding work with the purified ration (Sognmaes, '47, '48a) is mainly concerned. The present series of rat experiments has been started with a ration adequate in all known essentials. Many

TABLE 2
The particle size of the caries-conducive purified ration

COMPONENTS	QUANTITY	PERCENTAGE DISTRIBUTION OF PARTICLES (gm./100 gm sample)					
		Over 20 mesh	20-40	40-60	60-80	80-100	Below 100
	%						
Granulated cane sugar	67	0.3	39.2	42.4	12.0	3.4	2.7
Casein (and B-vitamins)	24	0	26.0	42.2	12.8	5.2	13.8
Salt mixture	4	2.2	18.1	12.5	4.3	5.5	57.4
Corn oil (and fat soluble vitamins)	5	0	0	0	0	0	(5)
All components	100	0.3	33	39	11	4	7 (& 5)
Portion requiring mastication	24	0 ¹	6	10	2	1	4

¹ This fraction constitutes 60% of the conventional caries producing coarse meal ration, the comparative particle size of which is reported elsewhere (Sognmaes, R. F.: Masticatory efficiency of rats (II), Am. J. Orthod. and Oral Surg., 27: 383-388, 1941).

factors which are present in natural foods, but whose nutritional importance is still unknown, are absent from the purified ration. This is in contrast to earlier rat caries work where the inclusion of powdered milk, alfalfa, linseed meal, whole corn, or rice particles (coarse rations) no doubt added unknown quantities of unknown trace elements, the confusing presence of which fortunately could be ruled out in this series of studies.

The purified ration has a physical composition such that it literally melts in the mouth. But for the sake of present or future comparisons with other caries producing rations, the particle size distribution has been expressed by actual measurements. It is shown in table 2 that the purified ration is essentially free from particles over 20-mesh and that a total of only 24% of the ration lends itself to some degree of mastication, while no fraction actually requires extensive mastication since it is readily dissolved by or suspended in saliva. The particle size of the previously used coarse caries producing diets has been analyzed elsewhere (Sognaes, '41). In these, the hard corn or rice particles, too large to pass a 20-mesh screen, represented 60% of the experimental ration (Hoppert, Webber and Canniff, '31). Particles over 20-mesh would correspond to a volume practically as large as the molar crowns of the rat. During the mastication of such a coarse ration, it was therefore not surprising that fractures of the molar cusp ensued, because 20-mesh particles would be too large to be properly masticated by the molars and, on the other hand, too small to be manipulated easily with the rat's forelegs and gnawed upon by the incisors.

Besides the absence of coarse food particles in the purified ration, the rats in the present as well as in the other most recent studies by the author were kept in screen bottom cages. Thereby the possibility is eliminated that accidental tooth fracture and food-impaction might be caused by the rat's mastication of wood shavings, the use of which as bedding has not always been stated but deserves consideration in the evaluation of this type of experiment. The latter precaution as well as the fine consistency of the purified ration would seem to satisfactorily rule out traumatic factors in the production of caries in animals kept under such a regimen. Nevertheless, in view of the wide acceptance in the past of a mechanical factor as the initial cause of rat caries, 2 experiments have been carried out, in which the parts played by the impact of mastication and the physical coarseness of the ration have been completely eliminated.

2. *Production of rat caries in absence of mastication
and coarse food particles*

In both experiments to be reported, representatives of the Long Evans strain of *Mus Norvegicus* were fed the purified ration described above. The carbohydrate fraction consisted of sucrose, in the form of granulated cane sugar, except for 1 series in the second experiment in which the sucrose fraction was replaced by dextrin. For the first experiment (table 3), a litter of 9 rats was weaned at 26 days of age, at which time the animals were transferred from the stock diet² to the purified ration for the duration of the experiment. When the rats were 30 days old, the mandibular right molars of each animal were relieved from the impact of mastication by an extraction method described elsewhere (Sognmaes, '41). The first and second right maxillary molars were extracted, while the third molar was left to maintain the occlusal level. In this way is prevented the remote possibility of any "occlusion" between the edentulous part of the maxillary process and the opposing first and second mandibular molars, which are the most caries susceptible teeth in the rat. With these precautions, it was assumed that a reliable comparison could be made between the mandibular right and left first and second molars within each animal with respect to the effect of the degree of masticatory impact on the caries production.

For the second experiment (table 4) 3 litters of 28 rats were used, representing 2 closely related groups which had been maintained on the purified sucrose ration for 1 and 2 generations, respectively. Littermates were divided at weaning, from which time one-half of the rats received the purified diet, and the other half was fed a diet which contained coarse dextrin instead of sucrose as the carbohydrate component of the purified ration. In this experiment, all animals had a full complement of teeth. The results are based upon a comparison of the caries incidence of littermates as revealed by the binocular examination. When sacrificed, the jaws of the

² Purina laboratory chow.

animals in both experiments were fixed in 10% formaldehyde, examined under a binocular microscope (30 X), and prepared for histological study.

In the extraction experiment (presented in table 3) a comparison is made between the distribution of grossly visible carious lesions in the right (experimental) and left (control) mandibular molars. In the 5 animals sacrificed after 75 days

TABLE 3

A comparison of the bilateral distribution of rat caries with and without masticatory impaction of the purified versus a coarse ration

PRE-EXPERIMENTAL PROCEDURE	EXPERIMENTAL		NO. OF CARIOUS AREAS IN LOWER JAW							
	Ration	Period	Left molars				Right molars			
			I	II	III	Total	I	II	III	Total
		<i>days</i>								
Group A	Purified	75	0	0	0	0	0	0	0	0
(The right	Purified	75	0	0	0	0	1	0	0	1
I and II	Purified	75	0	0	0	0	1	1	0	2
molars	Purified	75	0	0	0	0	1	1	0	2
of the	Purified	75	1	0	0	1	1	1	0	2
upper										
jaw ex-										
tracted)										
		75	(average)							1.4
	Purified	130	0	1	0	1	1	1	0	2
	Purified	130	1	1	0	2	1	1	0	2
	Purified	130	2	1	0	3 ¹	2	2	0	4 ¹
	Purified	130	2	2	0	4	1	1	0	2
		130	(average)							2.5
Group B ²	Coarse									
(The right	corn	100	0	1	0	1	0	0	0	0
I, II	Coarse									
and III	corn	100	0	1	0	1	0	0	0	0
molars	Coarse									
of the	corn	100	0	2	0	2	0	0	0	0
upper	Coarse									
jaw ex-	corn	100	1	2	0	3	0	0	0	0
tracted)	Coarse									
	corn	100	1	3	0	4	0	0	0	0
		100	(average)							0

¹ The carious lesions of this animal are shown in figure 1.

² This part is quoted from "Mastication and experimental rat caries" (Part IV) by Sognaes, R. F., Am. J. Orthod. and Surg., 27: 552-556, 1941.

on the purified ration, binocular examination revealed no visible carious destruction of the left molars. This finding is in agreement with the previously mentioned work in which rats with a full complement of teeth were fed for a similar period of time after weaning. In the right (experimental) molars, which were without functional occlusion, there were on the other hand 1-2 gross lesions in all but one of the same group of animals and obviously after the same period of time. Consequently, there appears to be an increment rather than a decrease in caries in the absence of masticatory function. In the littermates which were continued on the experiment, the number of carious lesions increased considerably beyond the incidence encountered in non-operated rats with a similar dietary history. After a total of 130 days, the caries incidence became equal and quite marked on both sides of the jaw. Figures 1 and 2 illustrate the very similar amount of carious destruction in the left and right mandibular molars, i.e., those with and without opposing teeth, respectively. The illustrations are from an animal which was marked and compared with its littermates in table 3.

For the sake of comparison, table 3 also records (group B) the distribution of lesions produced in rats fed a coarse corn ration used in an earlier extraction study which was similar except for the diets. On such a coarse ration, the removal of the impact of mastication completely prevented caries (Sogmaes, '41; Ginn, '42) while on the purified ration, the reduced masticatory function appears to have enhanced the initiation of carious lesions. The final bilateral distribution of caries may indeed have been influenced by a general impairment of function of the jaws. But aside from the caries incidence, there was no evidence that the impairment of normal masticatory efficiency affected the ingestion and utilization of the purified ration as far as could be determined by growth and general health.

The second experiment (table 4) shows that the inclusion of a coarser carbohydrate fraction in the purified ration is not caries conducive *per se*. In view of previous data ob-

tained with the coarse corn diet, consideration of the physical nature alone would indeed suggest the opposite. In the 15 rats fed the coarser dextrin ration, there was significantly less caries than in their 12 littermates fed the finer sucrose ration for a comparable period of time. This experiment lends further strength to the material presented elsewhere (Sognnaes, '47, '48a), on the basis of which it must be interpreted. There is a much greater cariogenic effect of sucrose than of dextrin when a change from one to the other is made post-eruptively. The great difference in caries in the 2 generations lends further support for a mechanism, operating before tooth eruption, and resulting in an increase in caries with the length of time of maternal maintenance on the purified ration. For our present purpose, it is evident that during such a period of non-function of the teeth, the mechanism of masticatory or traumatic injury of the enamel can have no conceivable direct bearing upon the initiation of caries. From the above experiments it may therefore be concluded that mechanical injury is a minor factor, if any, in the production of experimental rat caries of the nature reported by the writer in this and in the other most recent experiments with the purified ration.

3. Description of the carious lesions

Binocular examination of the carious molars (figs. 1 and 2) reveals a low degree of attrition, presence of undermining cavities and absence of fractures of the cavity walls and cusps. The high cusps are illustrated by the overall view of the section shown in figure 3, which is representative of a 3-month old rat, raised on the purified ration. In contrast to lesions

Figs. 1 and 2 Left (1) and right (2) mandibular molars of rat indicated by footnote 1 in table 3. Top: lingual view, showing absence of fractures and attrition in the non-functioning right first and second molars. Bottom: occlusal view, showing similarity in the extent of carious destruction in the left (control) and right (experimental) teeth.

Figs. 3 to 6. Gram stained paraffin sections, showing occlusal caries in the first and second molars (fig. 3, $\times 10$), spheroid organisms invading the enamel (fig. 4, $\times 1200$), lateral spread in dentin (fig. 5, $\times 200$) and microorganisms filling the dentinal tubuli and their branches (fig. 6, $\times 1000$).



Figures 1 to 6

TABLE 4

Litter mate comparison of molar caries in succeeding generations of rats with masticatory function intact but posteruptively fed the purified fine (sucrose) versus a medium-coarse (dextrin¹) ration

GROUP	PRE-EXPERIMENTAL HISTORY	POST-ERUPTIVE EXPERIMENTAL PERIOD	CARIOUS SCORE OF MOLARS POST ERUPTIVELY EXPOSED TO	
			Sucrose ration	Dextrin ration
		<i>days</i>		
I	Transferred from stock diet to purified ration at maturity	130	0	—
II (Litter of I)	Mother as well as litter subsisting on purified ration	100	0	—
		100	1	0
		100	2	0
		(average)	1	0
		130	2	—
		130	2	—
		130	4	0
		130	7	1
		(average)	3.7	.5
III (Litters of siblings of II)	Grandmother as well as mother and litters subsisting on purified ration	100	8	0
		100	8	0
		100	9	1
		100		1
		100		1
		100	9	0
		100	9	0
		100	12	1
		100	—	0
		100	—	2
		100	—	3
		(average)	9.1	.8

¹ In this ration the sucrose fraction was replaced by dextrin, which after soaking in water was dried and ground to particles, 40% of which were over 20 mesh.

produced by coarse diets, initial lesions in the enamel produced by the purified ration occur without fractures. Even on the lateral wall of the cusps, where fractures are very unlikely, regardless of the ration, there is evidence of early invasion of the enamel (fig. 4). In the described type of lesions, the dentine in the bottom of the cavities is usually very soft, more or less pigmented, and invaded by microorganisms. Following the dental tubuli and their branches, the process eventually spreads laterally causing large decayed clefts in the dentine (fig. 5). Under oil immersion, Gram stained sections reveal a spheroidal type of microorganism, both in the enamel (fig. 4) and in the furthest extension of the lesions into the dentine (fig. 6).

If the described lesions are compared with caries in man, the great similarity is obvious. Undermining cavities, soft pigmented dentine, lateral spread and cleft formation, invasion of the enamel as well as the dentinal tubuli by microorganisms with "pioneer-bacteria" invading the deepest layers, can be regularly demonstrated in the rat caries produced on the purified ration. Counterparts of the above description of the experimental lesions as well as the accompanying illustrations can be found in most textbooks on the histopathology of human caries.

COMMENT

In view of these results, the earlier type of rat caries produced by coarse rations seems to be explained by a coincidental choice of a food particle-size which could not be handled by the rats without mechanical injury to their molars. This does not detract from the importance of Hoppert, Webber and Canniff's work to the development of an experimental approach to the caries problem. Indeed, their method of caries production in the rat has been for over a decade a unique and useful *in vivo* adjunct to other means of elucidating factors influencing the progress of dental caries. As has been shown elsewhere (Hodge and Sogmaes, '46), this is particularly true of studies on the relationship of fluorine

to dental caries. But beside the artificial nature of "fracture caries" itself, the coarse corn rations by which these lesions are produced are composed of a variety of food sources of indefinite and indeed variable composition with respect to the known and unknown essential nutritional factors. In addition, no one essential nutrient can be experimentally varied through a wide range of concentrations independently of all other nutrients. By way of comparison, the composition of the purified ration is standardized and any nutrient can be independently altered at will. Thus, the purified ration permits the examination of the function of specific food factors in the etiology of caries which cannot readily be achieved by a diet composed of natural foodstuffs.

The pattern of caries production in rats fed the purified ration seems more suggestive of a nutritional mechanism operating during tooth development than of a purely physical mechanism operating during function of the teeth. Work is now in progress to test the importance to dental health of food factors not included in the otherwise adequate purified ration. For the present, it is difficult to conceive the nature of a nutritional depletion unless more evidence can be brought forth to show that the teeth are particularly sensitive (at least during their developmental period) to as yet unrecognized nutritional injuries. Therefore, at the present time, it is not possible to explain the results of our rat experiments by a deficiency in the amount or ratio of any group of nutrients, let alone by the lack of a specific trace element such as fluorine, the true significance and mechanism of which — as a caries modifier or as a nutritional essential — remains to be established.

SUMMARY

Evidence has been presented that a purified ration, adequate in known nutrients, is conducive to rat caries independently of the presence of coarse particles and the impact of mastication. Demonstration of a caries producing mechanism operating before tooth eruption, the absence of enamel

fractures and attrition, and the persistence of weakly supported overhanging enamel walls surrounding undermining caries, all tend to indicate that the described lesions are not caused by mechanical injury of the teeth. No difference can be found between the histopathology of the rat caries here presented and presently established findings in man.

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THE TOXICITY OF FLOURS TREATED WITH VARIOUS "IMPROVING" AGENTS¹

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For more than 25 years dog owners in this country as well as in England have observed in their animals the frequent occurrence of an affliction termed variously canine hysteria, fright fits, or running fits. The etiological agent has been demonstrated by Mellanby ('46) to be flour treated with an "improving" agent² known as agene. Agene is defined as 1% NCl_3 in air saturated with water vapor. The purpose of agene treatment is to alter the baking characteristics of freshly milled flour, thereby rendering it immediately usable in modern baking equipment, and eliminating the necessity of prolonged storage. The process has been in increasingly common use for over 25 years. While at the time of Mellanby's report an estimated 80% of the flour used for baking purposes in this country was agene-treated, the use of this agent has now been materially reduced.

¹ Presented before the Division of Agriculture and Food Chemistry at the 112th Meeting of the American Chemical Society on September 18, 1947, in New York, N. Y., and revised by the insertion of references to subsequent findings.

² The term "improving agents" is the technical designation in the milling industry for chemicals added to flour for artificial ageing and/or bleaching. In "Definitions and Standards for Food" (Federal Security Agency Service and Regulatory Announcement, Food, Drug, and Cosmetic no. 2) permitted optional ingredients for bleaching and/or ageing are oxides of nitrogen, chlorine, nitrosyl chloride, nitrogen trichloride, and benzoyl peroxide. Potassium bromate not in excess of 75 ppm is an optional ingredient in high protein flours.

Following the publication of Mellanby's original report, papers have appeared in this country and England both confirming his observation and extending the understanding of the phenomenon. Moran ('47) has shown that NCl_3 reacts with gluten, casein, zein and gelatin producing substances which give rise to canine hysteria, and further that the toxic principle cannot be separated from the protein fraction by extraction with organic solvents. These results have been confirmed by both Silver et al. ('47a,b) and Newell et al. ('47) who in addition have reported that monkeys exhibit abnormal encephalograms when fed agene-treated flour. Silver et al. ('47c) have also stated that the amino acids cysteine and cystine when treated with NCl_3 and administered intravenously to dogs produce the same electroencephalographic seizure patterns characteristic of fright fits. A second paper by Mellanby ('47) has shown ferrets to be susceptible to the actions of agenzized flour.

The work of this laboratory on the problem was begun soon after the appearance of Mellanby's original report and was directed toward the following objectives: (1) To determine if bread made from agene-treated flour is toxic; (2) to determine whether other treatments commonly used or suggested for use in bleaching or in improving the baking qualities of flour also render it toxic; and (3) to assess the possible hazard of agene-treated flour for human beings.

A brief description of the syndrome termed fright or running fits as produced in dogs by agene-treated flour is in order. The first observable symptoms are general lethargy and muscular incoördination. As the degree of poisoning becomes more advanced, a second stage of the disorder manifests itself in the form of attacks of hysteria in which the animal behaves in a thoroughly panic-stricken manner, throwing itself against the sides of the cage, clawing the air and howling piteously. If unconfined, it will run wildly about bumping into all objects in its path. These attacks may be of only fleeting duration or may last for a number of minutes. The dog usually recovers as suddenly as it is stricken. In very

severe poisoning, a third stage of the disease develops which is characterized by frequent epileptiform or tonic-clonic convulsions. These may become very severe and almost continuous until death occurs. In the experiments to be described, only the occurrence of the second or third stages, that is, actual fits or convulsions, was considered a positive result.

EXPERIMENTAL

Agene-treated flour and bread. Eighteen young mongrel dogs were divided into 4 groups. Group I consisting of 6

TABLE 1
Diets used in dog feeding experiments

	DIET A	DIET B
	<i>gm or ml</i>	<i>gm or ml</i>
Flour	150	220
or		
Bread ¹	225	
Milk powder	20	40
Liver	..	10
Horsemeat	15	..
Yeast	7.5	7.5
Salt	1	1
Corn oil	10	20
Baking powder	..	10
Cod liver oil	2.5	2.5
Water	150	150

¹ 225 gm of bread are the equivalent of 150 gm of flour.

dogs was fed flour with 1.4 gm of agene per 100 lb.; group II likewise consisting of 6 dogs was fed untreated flour; group III consisting of 3 dogs was fed bread made by standard baking procedures from the flour used for group I; and group IV also consisting of 3 dogs was fed bread similarly made from untreated flour. The diet used in this experiment was diet A in table 1. The rations containing flour were prepared daily by cooking in live steam for 90 minutes shortly before feeding. The diets containing bread were not cooked. The dogs were fed daily for a period of 6 weeks. They consumed

20 to 40 gm of flour per kilogram per day. The results are shown in table 2. All the dogs in group I fed agene-treated flour and those in group III fed the bread made from this flour developed typical fright fits in a period of feeding ranging from 7 to 17 days. It is clear that standard baking procedure does not diminish the toxicity of agene-treated flour.

*Flours treated with agene, chlorine dioxide, chlorine, oxides of nitrogen, benzoyl peroxide and potassium bromate.*³ Groups of 3 dogs each were fed diets containing flours which had been treated with 3 different levels of agene, 2 levels of chlorine dioxide, 2 levels of chlorine and 1 level each of oxides of nitrogen, benzoyl peroxide and potassium bromate. The

TABLE 2
Effects of feeding agene-treated flour and bread

PRODUCT TESTED	RATIO OF DOGS SHOWING FITS	AVERAGE OF DAYS BEFORE FIRST FIT
I. Agene-treated flour (1.4 gm/100 lb.)	6/6	15
II. Untreated flour	0/6	..
III. Bread made from agene-treated flour	3/3	17
IV. Bread made from untreated flour	0/3	..

degrees of treatment and the types of flour in each case are shown in table 3. The diet used was diet B in table 1, a slight modification of Mellanby's formula which we found more acceptable to our dogs. The preparation and feeding of the flours were the same as previously described. The daily consumption of flour ranged from 30 to 50 gm/kg/day. The results contained in table 3 demonstrate that of the group of "improving agents" tested only the agene treatment gave rise to fits or any other evidence of toxicity. The levels of treatment as shown in table 3 represent the upper limits of agene treatment commonly used for clear, straight and patent flour, respectively. There is some suggestion that the heavily

³ These flours were prepared under commercial conditions in the presence of Inspectors of the Food and Drug Administration.

treated clear flour was more toxic than the lightly treated patent flour.

The acute toxicity of agene treated gluten. In an attempt to prepare a product having a consistently high degree of toxicity which would be suitable for acute toxicity studies, commercial gum gluten (80% protein)⁴ was treated directly with large quantities of agene. The agene was prepared by

TABLE 3

Effect of feeding dogs with flours treated with various improving agents

AGENT	TYPE OF FLOUR	DEGREE OF TREATMENT	RATIO OF DOGS SHOWING FITS	DAYS BEFORE FIRST FIT
Agene	Clear	3 gm/100 lb.	2/3	20 & 23
	Straight	2 gm/100 lb.	2/3	30 & 35
	Patent	1 gm/100 lb.	1/3	20
Chlorine	Straight	0.5 oz./100 lb.	0/3	..
	Patent	1 oz./100 lb.	0/3	..
Chlorine dioxide	Straight	0.6 gm/100 lb.	0/3	..
	Straight	1.8 gm/100 lb.	0/3	..
Oxides of nitrogen	Straight	11 amperes/100 lb.	0/3	..
Benzoyl peroxide	Straight	1 oz./100 lb.	0/3	..
Potassium bromate	Straight	3.4 gm./100 lb.	0/3	..
Untreated	Straight	0/9	..
	Patent	0/3	..

the method customarily used in commercial practice, i.e., by bubbling chlorine into an aqueous solution of ammonium chloride. The NCl_3 was then aerated from the solution and conducted into a revolving, motor driven box containing the gluten. To obtain a product of maximum toxicity the gluten was treated with increasing amounts of agene until no further appreciable increase in toxicity occurred. With

⁴ A product of the Keefer Starch Company, Columbus, Ohio.

this heavily treated gluten typical fits and convulsions were obtained when single doses were administered to dogs. Table 4 contains the acute toxicity data which were obtained. Increasing the degree of treatment from 1.5 gm of agene/1000 gm of gluten to 3 gm of agene/1000 gm of gluten increased the toxic effect decidedly. However, doubling the degree of treatment again produced no further significant increase in toxicity. Therefore, saturation was achieved somewhere between the 3 gm/1000 gm and the 6 gm/1000 gm treatment. It must be remembered that the use of the term "saturation" refers only to maximum effect obtained under

TABLE 4
Acute toxicity of agene-treated gluten to dogs

DEGREE OF AGENE TREATMENT	GLUTEN ADMINISTRATION		RATIO OF DOGS SHOW- ING FITS	MORTALITY RATIO
	Dose	Mode		
<i>gm/kg</i>	<i>gm/kg</i>			
1.5	15	Fed	2/3	0/3
3	15	Fed	4/4	3/4
3	5	Stomach tube	1/2 ¹	0/2
3	2	Stomach tube	0/2	0/2
6	5	Stomach tube	2/2 ¹	0/2
6	2	Stomach tube	0/2	0/2

¹ Dogs in both groups showed fits of equally mild intensity.

the conditions of treatment and the particle size of the starting material. It is not meant to imply that further increases in toxicity could not be obtained under different conditions. It is also apparent from these data that the ED 50 (the amount of a substance which produces an effect in 50% of the animals) of this agenized gluten is about 3.5 gm/kg.

Comparative toxicity of other proteins. If, as reported by other investigators, agene reacts with casein and gelatin to yield the same toxic principle which produces fright fits, a comparison of the acute toxicity of these agene-treated proteins would be informative. Highly purified casein and gelatin were treated with the same high level of agene (6 gm/1000 gm) as produced a maximum effect with gluten. These substances

were fed to fasted dogs after mixing with equal quantities of sugar and enough corn oil to make the mixture palatable. The results are contained in table 5. Significantly, agene-treated casein is as potent a fit-producer as agene-treated gluten, while gelatin did not produce fits in the dosage administered. These results obtained with gelatin are not in conflict with those reported by Moran ('47), for this investigator obtained positive results only after a prolonged period of feeding.

In addition, treated gluten was fractionated by extraction with 70% alcohol into its glutenin and gliadin components.

TABLE 5
Acute toxicity of agene-treated proteins to dogs

AGENT	DOSE	RATIO OF DOGS SHOW- ING FITS	MORTALITY RATIO
	<i>gm/kg</i>		
Gluten	15	4/4	3/4
Casein	15	2/2	2/2
	5	2/2	0/2
Gelatin	100	0/2	0/2
Glutenin	7.5	1/2	0/2
Gliadin	7.5	2/2	1/2

Both these fractions produced fits on feeding but the gliadin appeared to be the more toxic.

Because of the possibility that a simple chemical alteration of a constituent amino acid may be responsible for the toxicity of agene-treated flour, several amino acids in dry form and in solution were treated with agene. These substances were administered by stomach tube to dogs in doses several times larger than the amount of the particular amino acid which would be present in a toxic dose of maximally toxic gluten. No toxic effects were obtained with agene-treated tryptophane, acetyl-tryptophane, tyrosine, methionine, cysteine or cystine. A pancreatic digest of agene-treated gluten was also prepared. Fits and convulsions were obtained when fasted dogs were given 100 ml/kg by stomach tube. (Ten ml con-

tained the products of digestion of 1 gm of gluten.) When the insoluble fraction containing the tyrosine was fed to dogs, no toxicity was obtained.

Variations in species susceptibility. Because toxicity data on a variety of species of animals are of paramount importance in evaluating the possible human hazard, agene-treated gluten was fed or administered by stomach tube to rats, rabbits, monkeys, guinea pigs and cats. The guinea pigs, rabbits and cats received by stomach tube a 25% suspension of 6 gm/1000 gm agene-treated gluten; the monkeys were fed a diet containing 30% agene-treated gluten; the rats were given a diet consisting solely of 1.5 gm/1000 gm agene-treated

TABLE 6
Susceptibility of various species to agene-treated gluten

ANIMAL	DOSE	NO. OF DOSES	MODE OF ADMINISTRATION	NO. OF ANIMALS	RESULTS
	<i>gm/kg</i>				
Dogs	5	1	Stomach tube	4	Fits
Rats	42	30	Fed	6	No fits
Monkeys	15	54	Fed	2	No fits
Guinea pigs	15	1	Stomach tube	10	No fits
Rabbits	15	1	Stomach tube	8	Fits
Cats	15	3	Stomach tube	4	Fits

gluten. The results obtained are shown in table 6. Most noteworthy is the observation that rabbits are of a similar order of susceptibility as dogs. Within 48 hours after the administration of a single dose of 15 gm/kg, rabbits developed fits and convulsions strikingly similar to the syndrome obtained with dogs. Cats developed similar symptoms after 3 administrations. Rats and Rhesus monkeys, however, did not develop any observable central nervous disorders over the prolonged period during which they were fed these large amounts of this exceedingly potent gluten. Guinea pigs did not develop symptoms from a single administration.

The observation of the high degree of susceptibility of rabbits to the effects of agene-treated gluten presents the at-

tractive possibility of their use as an assay animal, not only in testing the potency of various agene-treated proteins, amino acids and other synthetic substances, but also in following the toxicity of fractions obtained during the isolation of the toxic principle from hydrolysates.

DISCUSSION

The question arises as to whether the agene treatment acts by destroying a necessary dietary constituent of flour or by producing a toxic product. The fact in itself that the typical running fit syndrome can be produced in dogs on an acute basis very strongly suggests that the problem is one of simple toxicity uncomplicated by dietary deficiencies.

The question of the identity of the agent responsible for the production of fright fits in dogs remains largely unsettled. That the toxic principle of agene-treated flour is associated with the protein fraction seems to be adequately established. Since proteins are broken down in the animal body and gain access to the blood stream largely as amino acids, it seemed likely that the toxic principle would be a chemically altered amino acid. The report by Silver et al. ('47c) that intravenously administered agene-treated cystine and cysteine will produce characteristic encephalographic seizure patterns in dogs would indicate that the toxic principle was a reaction product of these amino acids and NCl_3 . If this were true, the acute oral administration of these agene-treated acids in large doses should have produced a toxic effect. Completely negative results were obtained. However, the possibility still remains that the sulfur-containing amino acids are somehow more complexly involved in the reaction as, for instance, part of a dipeptide. The possibility also remains that an amino acid not yet tested may be responsible. The fact that an enzymatic hydrolysate retained all the toxicity of the agene-treated protein from which it was prepared again suggests an amino acid or a low molecular weight polypeptide. That the toxic principle is a di-, tri- or poly-peptide absorbed intact into the animal's blood stream seems most likely at the present.

Although the data presented definitely demonstrated that the production of fright fits by the agene-treated gluten of flour is not a phenomenon peculiar to the dog but rather a toxic reaction common to several species, the question of possible toxicity to human beings is still unsettled. Obviously a toxicant of such potentialities for harm is not a desirable constituent of the flour from which our daily bread is prepared.

SUMMARY

1. Bread made from agene-treated flour produces fright fits in dogs.

2. Oxides of nitrogen, benzoyl peroxide-, chlorine-, chlorine dioxide- and bromate-treated flours are harmless to dogs when fed for 6 weeks.

3. The ED 50 of gluten "saturation-treated" with NCl_3 is approximately 3.5 gm/kg.

4. The reaction product of NCl_3 and either tyrosine, tryptophane, cystine, cysteine or methionine is not responsible for the production of fright fits.

5. Rabbits are of the same order of sensitivity to agene-treated gluten as dogs and present attractive possibilities for use as assay animals.

6. Cats are also sensitive but less so, while rats and Rhesus monkeys are apparently resistant insofar as the production of gross symptoms is concerned.

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PROLONGATION OF THE LIFE SPAN OF RATS BY BULK-FORMERS IN THE DIET¹

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McCay ('29, '34) found that rats fed a diet including 10% ground no. 300 glycerine-free cellophane were considerably retarded in growth but lived longer than those fed a stock diet; they also lived longer than rats raised by Campbell ('28) on the superior growth-promoting diet of Sherman and Campbell ('24). The longer life span of the cellophane-fed rats was attributed by McCay ('33, '34) to the retardation in growth rather than to the cellulose in the diet. However, our observations concerning the effect of various types of bulk-formers (Hoelzel and Da Costa, '37; Hoelzel, '39; Hoelzel, Da Costa and Carlson, '41) indicated that ground cellophane was too irritating for liberal use in the diet and suggested that a diet including about 10% of a more suitable type of bulk-former would be likely to prolong the life span of rats without a significant retardation of growth. It was also thought that the roughage in natural foods might serve to prolong life without significantly retarding growth. These possibilities were investigated in connection with our 2 studies of the effects of intermittent fasting and omnivorous and vegetarian diets on the growth and longevity of rats (Carlson and Hoelzel, '46, '47).

¹ This study was aided by a grant from Swift & Company, Chicago.

FIRST LONGEVITY STUDY

Diets used and experimental procedures

Some details concerning our diets and experimental procedures were given in our preceding reports. In our first longevity study, diet 2 (table 1) was used to determine the

TABLE 1
*Composition of diets*¹

DIET ²	
1	Basal or low residue omnivorous diet including 61.5% dried "whole veal," ³ 3% veal bone meal, ⁴ 31% cornstarch, 2% dried brewers' yeast, 1.5% inorganic salt mixture and 1% cod liver oil.
2	Diet 1 plus 10% finely ground alfalfa stem meal.
3	Diet 1 plus 5% ground kapoc ⁵ and 5% psyllium seed husks. ²
4	Simple vegetarian diet including 50% whole wheat flour, 10% peanut flour, 7% wheat gluten flour, 7% lima bean flour, 7% linseed meal, 7% corn gluten meal, 5% alfalfa leaf meal, 5% brewers' yeast and 2% NaCl.
5	Diet 1 plus 10% semi-fibrous cellulose flour. ^{6, 7}
6	Vegetarian self-selection diet including corn (whole kernels), wheat (whole grain), pearled barley, rolled oats, sunflower seeds, peanuts, green peas, soy beans, corn germ meal, wheat germ meal, brewers' yeast, alfalfa leaf meal and NaCl.
7	Modification of diet 3 with 5% semi-fibrous cellulose flour ⁶ in place of 5% ground kapoc. ²
8	Diet 1 plus 10% granular (80 mesh) karaya gum ⁷ gradually increased to 25% and granular gum acacia ⁷ used in place of karaya gum during 2 months.
9	Modification of diet 7 — diet 1 plus 6.7% semi-fibrous cellulose flour and 3.3% psyllium seed husks. ²
10	Diet 1 plus 10% ground (40 mesh) glycerine free no. 150 cellophane. ⁷
11	Diet 1 plus 20% semi-fibrous cellulose flour and 5% psyllium seed husks.
B	Refers generically to diets with 10% added bulk (diets 3, 5, 7, 9 and 10).

¹ Trimmings of head lettuce were fed ad libitum as a supplement to all diets.

² Changes in diet are indicated by hyphenated figures. Thus, diet 3-7-9 indicates a change from diet 3 to diet 7 and finally to diet 9. In general, only 1 change was made, such as in diets 1-B, B-1 and 5-10.

³ Especially prepared for us by Swift & Company, Chicago.

⁴ Obtained from Mead Johnson & Company's laboratory.

⁵ Freed from seeds, boiled, washed, partly bleached and dried.

⁶ Cellu Flour B.

⁷ Obtained from The Chicago Dietetic Supply House.

effect of a natural source of roughage and an amount of roughage, added to our basal omnivorous diet (diet 1), that served to produce approximately the same volume of feces as our simple vegetarian diet (diet 4). Diet 3 was used to determine the effect of a type of bulk-former regarded as more satisfactory than the ground cellophane used by McCay (Hoelzel, '39) and fed in an amount approximately equal to 10% cellophane. In this study, 45 rats were fed diet 1, and 2 groups of 23 each were given diets 2 and 3, respectively. Besides this, separate life span observations were made on 6 second generation rats on diet 2. Approximately equal numbers of the rats were fed the diets ad libitum or fasted 1 day in 4, 3, or 2.

Results

The rats fed diet 2 did not gain weight as rapidly as the animals on diet 1, but they gained weight more rapidly than those fed diet 3. The maximum weights attained, femoral lengths and ages at death of the rats are presented in table 2, but not the specific effects of the different degrees of fasting. Although the rats fed diets 2 and 3 did not become as heavy as the animals fed diet 1, they attained the same size (femoral length). The rats on diet 2, including the 6 second generation rats on this diet, did not live longer than those on diet 1, but the animals on diet 3 lived longer (average, 77 days longer). Most of the fasted rats on diets 2 and 3 lived longer than those fed ad libitum, but the precise effect of fasting could not be clearly evaluated from the limited amount of data.

A search for lower bowel lesions revealed no gross evidence of chronic or ulcerative cecitis but diverticula of the colon near the cecum, such as Wierda ('43) discovered, were found, particularly in rats on diets 1 and 2. Details will be reported in a separate paper.

The females on diets 2 and 3 did not develop as large mammary tumors as those on diet 1 but there was no significant difference in the proportion of females developing this condition.

TABLE 2

Maximum weights, femoral lengths, and ages at death of rats fed a low residue omnivorous diet (diet 1), this diet with added bulk-formers (diets 2, 3, 5, 7, 8, 9, 10 and 11) and a vegetarian self-selection diet (diet 6), ad libitum or with intermittent fasting during 2 longevity studies

DIET ¹	NO. OF RATS		MAXIMUM WEIGHT ²		FEMORAL LENGTH ²		AGE AT DEATH ³	
	♂	♀	♂	♀	♂	♀	♂	♀
			gm		mm		days	
FIRST LONGEVITY STUDY								
Rats fed ad libitum								
1	5	7	591	456	39.0	34.7	633 ± 123	659 ± 125
2	2	3	566	389	40.3	35.0	552 ± 147	785 ± 102
3	2	3	466	453	39.4	35.2	661 ± 145	742 ± 19
Rats fasted 1 day in 4, 3 or 2								
1	14	19	407	325	38.1	34.8	667 ± 143	758 ± 114
2	7	11	383	285	38.3	34.6	637 ± 113	735 ± 200
3	9	9	399	292	38.5	35.3	738 ± 191	842 ± 201
SECOND LONGEVITY STUDY								
Rats fed ad libitum								
1	4	3	639	551	39.8	33.2	588 ± 96	701 ± 129
3-7-9	2	3	592	441	38.3	34.6	718 ± 38	751 ± 122
5	5	4	668	421	37.9	35.3	807 ± 191	646 ± 61
10	1	1	674	336	38.6	32.9	842	418
5-10	2	2	758	397	38.7	34.2	691 ± 5	692 ± 141
1-B	3	2	649	453	39.1	33.3	864 ± 175	911 ± 127
B-1	4	4	658	448	37.8	34.3	687 ± 57	850 ± 211
1-11	2	1	506	316	38.3	34.4	906 ± 158	937
B-11	5	5	497	398	37.4	33.8	692 ± 243	937 ± 72
8	2	1	650	410	38.2	34.4	920 ± 11	779
1-6	1	1	524	362	40.9	34.3	564	585
B-6	1	2	576	422	35.9	34.4	718	853 ± 119
6-1	1	1	570	244	37.8	32.1	511	379
6-B	3	3	572	383	38.2	34.8	845 ± 262	826 ± 95
Rats fasted 1 day in 3								
1	3	2	537	355	38.0	33.3	676 ± 136	930 ± 26
3-7-9	2	2	502	427	38.5	34.9	630 ± 220	840 ± 44
5	3	4	531	398	37.7	34.6	730 ± 183	972 ± 124
10	1	1	512	430	37.7	35.9	845	777
5-10	1	2	550	366	38.2	34.4	863	983 ± 105
1-B	1	2	470	402	38.0	34.0	636	808 ± 95
B-1	2	4	495	393	37.8	34.0	715 ± 132	935 ± 25
8	1	2	450	341	36.8	33.3	708	867 ± 20
1-6	1	1	458	330	38.3	32.9	470	835
B-6	2	1	446	282	37.6	33.9	550 ± 73	645
6-1	0	0						
6-B	1	2	436	262	37.7	32.9	798	799 ± 383

¹ See table 1, footnote 2, concerning changes in diets.

² Mean.

³ Mean and standard deviation.

Discussion

The failure of the rats on diet 2 to live longer than those on diet 1 in spite of their lower maximum weights was apparently partly due to the unsatisfactory nature of alfalfa stem meal as a source of roughage. It seemed that it introduced some deleterious substance(s) which increased the incidence and severity of respiratory infections. Alfalfa stem meal also did not seem to provide enough hemicellulose to keep the feces from becoming too firm for easy passage. Wheat bran might have served as a better natural source of roughage, but alfalfa stem meal was used in preference to wheat bran because it introduced less vegetable protein into the diet which was intended to contain mainly meat protein. However, even if a more satisfactory source of roughage than alfalfa stem meal had been used, the amount of roughage provided would not have been likely to increase the life span significantly. This is suggested by the results obtained with diet 3 which provided at least twice as much of a more satisfactory type of bulk and apparently introduced nothing of a deleterious nature. Although the rats on diet 3 lived, on the average, 77 days longer than those on diet 1, more data seemed needed to prove that this extension of the life span was statistically significant. A second study was therefore begun in which the main object was to secure more data on the effect of various kinds and amounts of relatively pure bulk-formers on growth and longevity.

SECOND LONGEVITY STUDY

Diets used and experimental procedures

Some details concerning the diets and experimental procedures in our second longevity study were also given in a preceding report ('47). In this study, diets 3, 5, 7, 8, 9, 10 and 11 (table 1) were used to secure data on the effect of bulk-formers added to diet 1. Diets 3, 5, 7, 9 and 10 were alike in involving the addition of 10% bulk to diet 1 and these diets are therefore generically referred to in some connections as diet B. Diet 3 was replaced by diet 7 because the supply of kapoc

was cut off by the war and, later, diet 7 was replaced by diet 9 to conserve the supply of psyllium seed husks. Rats fed these diets are therefore referred to as rats on diet 3-7-9. Diet 5 served to determine the effect of the cellulose flour used in diets 7 and 9 without added hemicellulose in the form of psyllium seed husks. Diet 10 served to determine the effect of a form of cellulose less irritating than the ground no. 300 cellophane used by McCay: the no. 150 cellophane used here was only half as thick as no. 300 cellophane. Diet 11, with 25% added relatively smooth bulk, was used to compare its effect with McCay's ('34) use of a diet including 20% ground no. 300 cellophane. Diet 8 was used mainly to determine whether karaya gum would produce ulcerative cecitis in the (Wistar) rats that we were using as they did not develop ulcerative cecitis "spontaneously" like the rats that we had used in a previous test of the effect of karaya gum (Hoelzel, Da Costa and Carlson, '41). Gum acacia was used in place of karaya gum in diet 8 during a period in which suitable karaya gum was not obtainable.

Eighteen litters, including 115 rats, were used in this study. The original intention was to use a much larger number of rats but wartime and other conditions beyond our immediate control prevented this. Five of the 18 litters were started on diet 1, 4 on diet 3-7-9, 6 on diet 5, 1 on diet 10 and 2 on the vegetarian diet 6. However, 2 of the 6 litters started on diet 5 were so-started merely in preparation for being placed on diet 10 when about 100 days old. Fourteen rats started on diet 1 and 16 started on diet B (diets 3-7-9, 5, 10 and 5-10) were kept on these diets throughout life. Nine rats started on diet 1 were placed on diet B when 72 to 200 days old. Six rats (1 male and 1 female from each of 3 litters started on diets 1, 5 and 6) were placed on diet 8 when 42 days old. Eight males and 7 females started on diets 1 and B were placed on diet 11 when 147 to 200 days old. All rats started on the vegetarian diet 6 were placed on diet 1 or diet B before they became 345 days old and 10 rats started on diets 1 and B were placed on the vegetarian diet when 72 to 200 days old. All rats on diet

11 were fed ad libitum throughout life. About half (45) of the other 100 rats in this study were fasted 1 day in 3 after they became 100 to 200 days old.

The results were statistically evaluated by calculating *t* values according to the small sample methods explained by Fisher ('37) and Snedecor ('46) and probability (P) values of less than 5% were regarded as indicating significant differences.

Results

Growth curves showing the weights between the ages of 42 and 200 days of the rats fed diets 1, B and 6 ad libitum were included in our preceding report ('47). The maximum weights attained, femoral lengths, and ages at death are presented in table 2. However, data on 5 rats that died relatively early and apparently because of factors other than diet or fasting are not included in table 2. One of the 5 excluded rats was an intermittently fasted male on diet 5-10. This animal died of a rapidly growing tumor on the nape of the neck when 358 days old. The other 4 excluded rats were 2 males and 2 females that constituted all of the young in the last (7th) litter of one of our breeding females that were reared. This litter was started on diet 1 and kept on this diet until 147 days old. After that, 1 male and 1 female were intermittently fasted and the other male and female were placed on diet 11. The males died when 329 and 345 days old and the females when 477 and 522 days old, respectively. In each case, the rat fed diet 11 lived longer than the fasted litter mate kept on diet 1. The rats raised in the last litters of our 2 other breeding females that were mated with the same male lived to be 635 to 1018 days old (6 males) and 741 to 937 days old (3 females), respectively. One of these litters, including 4 males and 3 females that were reared, also had been started on diet 1. A male of this litter placed on diet 11 when 150 days old lived to be 1018 days old, and a female on the same regimen became 937 days old. The exclusion of data on the 4 rats in the short-lived last litter therefore seems amply justified.

The data in table 2 indicate that the average weight attained by the males fed diet B ad libitum was greater than that of the males fed diet 1 ad libitum in spite of the fact that growth was slower on diet B. The females on diet B, however, did not become as heavy as those on diet 1. This sex difference in attained weights seems largely explainable by the effects of the diets on the life spans. The males on diet B lived longer than those on diet 1 (average, 181 days longer — $P < 5\%$). Obviously, the longer life span of the males on diet B included a longer period during which growth continued. The females on the other hand, particularly those on diets 5, 10, and 5-10, did not live longer than those on diet 1 and therefore did not attain similar weights. The skeletal growth (femoral length) of the males started early on diet B appears to have been stunted slightly; this was not true of the females. Both males and females fed diet 1 early in life and diet B later in life (diet 1-B) lived longer than those fed either diet ad libitum throughout life. The data on the reversed regimen (diet B-1) only indicate that the opposite regimen is superior. The rats fed diet 11 after diet 1 or B (diet 1-11 or B-11) lived longer than those kept on diet 1 or B. This is not indicated by the data in table 2 concerning the males on diet B-11, but 4 of the 5 males had litter mates that were kept on diet B and all of the litter mates fed diet 11 lived longer (average, 83 days longer). The females on diet B-11 lived much longer than those kept on diet B (average, 273 days longer — $P < 1\%$) but this difference is partly explainable by the relatively short life spans (average, 664 days) of the females kept on diet B.

The life spans of the rats fed diet 8 were similar to those of rats fed other bulk-formers but the karaya gum appeared to have a depressive effect on the rats. This seemed to be partly due to a reduction of the food intake because of the unpalatability of the gum. It was originally intended to use only 10% (added) karaya gum, but the volume of feces produced was very small. Even 25% karaya gum did not produce very voluminous feces, presumably because of the relatively

low food intake and a breakdown of the gum in the digestive tract. The food intake increased and the weights increased rapidly when the more acceptable gum acacia was used. The alertness of the rats also increased then but the volume of feces decreased because of an even greater breakdown of gum acacia in the digestive tract.

TABLE 3

Summary of effects of diets fed ad libitum on life span of rats in second longevity study

DIETS	MALES		FEMALES		SUITABILITY OF DIET
	No.	Age attained ¹	No.	Age attained ¹	
Omnivorous					
3-7-9, 1-B, 1-11, B-11, 8	14	795 \pm 192	12	873 \pm 116	Relatively satisfactory for both sexes
1, B-1	8	638 \pm 90	7	786 \pm 196	Less satisfactory for males than females
5, 10, 5-10	8	782 \pm 156	7	626 \pm 119	Less satisfactory for females than males
Part-time vegetarian					
B-6, 6-B	4	813 \pm 223	5	836 \pm 91	More satisfactory for both sexes than diets 1-6 and 6-1
1-6, 6-1	2	538 \pm 38	2	482 \pm 146	Less satisfactory for both sexes than diets B-6 and 6-B

¹ Mean and standard deviation.

Tables 2 and 3 show that the rats fed diet B before or after the vegetarian self-selection diet (diets B-6 and 6-B) lived longer than those fed diet 1 before or after the vegetarian diet (diets 1-6 and 6-1).

The intermittently fasted males on diet B did not live longer than those fed diet B ad libitum. The intermittently fasted females on diet B lived longer (average, 259 days longer — $P < 1\%$) than those fed diet B ad libitum, but this great

difference is again largely due to the relatively short life spans of the females fed diet B ad libitum.

No grossly evident cecal lesions were found in the rats on diet 8 but further evidence was found that the development of diverticula of the colon was promoted more by the low residue diet than by the bulky diets. Differences in the effects of the different bulk-formers were nevertheless also noted and the details will be reported in a separate paper.

The incidence of mammary tumors did not seem to be reduced by the bulky diets. In this study, also, the size attained by the tumors was not clearly reduced by diet B (10% added bulk) but it was definitely reduced by diet 11 (25% added bulk).

Discussion

A further study will be needed to explain the differences in the effects of the different bulk-formers on the 2 sexes and under different conditions. The differences in rats fed ad libitum are clearly indicated in table 3. Comparisons between litter mate male and female rats on diet B showed that the males fed ad libitum lived 174 days longer than the females fed ad libitum but that the intermittently fasted females lived 158 days longer than the fasted males ($P < 5\%$ in both comparisons, which involved 8 and 7 pairs of rats, respectively). The complicated nature of the sex difference is further emphasized by the fact that the 5 females on diet B-11 (without fasting) lived as long as their intermittently fasted litter mate females on diet B. This was also true of 5 additional pairs of females, of which 1 of each pair was started or continued on diet 1 and the other was placed on diet 11, and 10 pairs of litter mate males started on diets 1 and B. This incidentally indicates that diet 11 (sufficient bulk but including psyllium seed husks) fed ad libitum served as well as fasting 1 day in 3 in prolonging the life spans.

The types and amounts of bulk-formers that would serve best at different ages to promote the greatest length of life without stunting growth or impairing vigor also need further clarification. Our results suggest that a suitable type and

proportion of hemicellulose as well as cellulose should be included in the diet and that no bulk-former or only small and gradually increasing amounts should be used at early ages. Psyllium seed husks seem to provide a suitable type of hemicellulose. We did not determine the effect of using psyllium seed husks alone because the mucilage which the husks alone form cannot be segmented normally in the digestive tract. The mixtures of kapoc or cellulose flour and psyllium seed husks which we used were apparently segmented normally but the proportions that might serve best remain unsettled. Our observations suggest that as much as 10% bulk should not be used before rats are about 150 days old. Diet 11, with 25% added bulk, was evidently also too bulky for some rats 200 days old, but it seemed to be satisfactory for the rats when they were over 500 days old. This may have been partly due to the naturally reduced food intake with ageing. The life span of some of our bulk-fed rats, may therefore have been prolonged partly by a slowing down of living processes during some periods by the diets being too bulky to permit a sufficient caloric intake, but we believe that the life span of most of our bulk-fed rats was prolonged without any slowing down of living processes. During middle life, some of the rats on diet B were unquestionably more active or alert than any rats on diet 1. This may, of course, merely mean that the activity of the rats on the low residue diet was depressed. On the other hand, the prolongation of the life span of the rats fed the bulky diets *ad libitum* was evidently not due to increased activity such as often occurs on the days of fasting in intermittently fasted rats.

The food intake of our rats was not determined, but large amounts of non-nutritive materials in the diet obviously reduce the caloric intake by filling the digestive tract and dispelling the desire to eat with the ingestion of less nutriment (Hoelzel, '47). Theoretically, the food intake with *ad libitum* feeding can be reduced to any desired level by simply including the necessary proportion of inert material in the diet, but the optimum amount of a bulk-former that can be included

in the diet depends on the nature of the bulk-former and the nutritional requirements for normal growth and the maintenance of vigor. It is evidently more practical to prevent the ingestion of excessive amounts of nutriment by feeding a bulky diet ad libitum than by intermittent fasting or by feeding restricted amounts of a relatively low residue diet daily. The possibility also exists that suitable bulk-formers help to prolong life by normalizing intestinal conditions. This is suggested partly by the finding that colonic diverticula were absent in practically all rats fed the diets including psyllium seed husks.

Our second life span study was designed partly to secure data concerning the relative influence of heredity and litter order as well as nutrition on the life spans but much more data than we secured would be needed for such a purpose. Nevertheless, the relatively early death of all of the rats in one of the 3 last litters suggests that last litters or offspring of ageing rats are not likely to live as long as offspring of younger rats. First litters were not used in our second study but the data obtained on second to last (6th, 7th and 8th) litters indicated that the constitutions or potential life spans of the rats in litters after the 4th were at least more frequently poor than among the rats in the earlier litters. Data on successive generations, such as Lansing ('47) obtained on rotifers, would evidently be necessary to determine the specific effect of ageing on the life span of offspring.

The failure of cecal ulceration to develop in the rats fed karaya gum in this study suggests that this gum merely increased "spontaneous" cecal ulceration in the rats used in our previous studies (Hoelzel, Da Costa and Carlson, '41, and unpublished observations). In short, the view of Bloomfield and Lew ('43) that ulcerative cecitis in rats is of infectious origin is indirectly supported. However, the fact remains that karaya gum and apparently also some other bulk-formers increase ulcerative cecitis in infected rats.

SUMMARY

The effects of feeding a low residue diet and this diet with added bulk-formers on the growth and longevity of 212 Wistar rats were determined. The bulk-formers used included alfalfa stem meal, psyllium seed husks, ground kapoc, cellulose flour, ground cellophane and granular karaya gum. The effects of feeding the different diets ad libitum or with intermittent fasting, and of feeding the low residue or bulky diets early or late in life were simultaneously investigated.

The results indicated a sex difference in the effect of bulk-formers. In rats fed ad libitum the life span of both sexes was increased by 10% and 25% (added) relatively smooth bulk-formers but the life span of females was not increased by 10% cellulose flour or ground cellophane. The results differed in intermittently fasted rats and in those fed different amounts of bulk during different periods of life. No impairment of skeletal growth was produced when diets with 10% bulk-formers were fed only after the rats became 42 or more days old. A diet with 25% relatively smooth bulk prolonged the life span as much as fasting 1 day in 3, which was previously found to be the optimum amount of intermittent fasting for rats.

Contrary to findings in previous studies, no cecal ulceration was found in 6 rats fed 10% to 25% granular karaya gum.

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THE TOTAL SPECIFIC DYNAMIC ACTION OF HIGH-PROTEIN AND HIGH-CARBOHYDRATE DIETS ON HUMAN SUBJECTS ¹

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TWO FIGURES

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The estimation of the fuel value of human diets by the application of the Atwater factors of 4, 4 and 9 cal. per gram of total protein, carbohydrate and fat, respectively, allows for average losses of food energy in digestion, and the incomplete oxidation of protein in metabolism under conditions of nitrogen equilibrium. It does not, however, take account of the stimulating effect of the food on metabolism, the specific dynamic action. The energy thus dissipated, except under environmental conditions otherwise tending to induce a negative heat balance, is a wastage of food energy as far as its value in promoting physiological work is concerned. Allowance must, therefore, be made for this wastage of energy in computing the amount of food energy required for persons of given size, age and activity. Hence, the amount of this wastage is of importance in practical dietetics.

¹The data presented in this report were secured in an experimental project covered by a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Illinois.

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For this purpose, the specific dynamic action (S.D.A.) of food should be measured as the total extra heat produced post prandium above a baseline level. Of the many investigations that have been reported on the specific dynamic effect of food in humans, relatively few involve the measurement of the extra calories above basal produced by a meal of definite description, and very few indeed extend over a sufficiently long period of time post prandium to measure the total effect. The most complete study of this nature was reported by Benedict and Carpenter ('18), involving many experiments and many subjects. The general conclusion from this monumental work was that the specific dynamic action of pure carbohydrates and predominantly carbohydrate meals amounted to about 6% of the fuel value of the food ingested; for fats, the average value is about 2%, while with a protein-rich diet, the S.D.A. approximates 12% of the metabolizable energy (fuel value) ingested. For a mixed diet, "which more properly corresponds with every-day usage," a value of 6% is deduced from the experimental data. Concerning these results, the authors say ". . . in drawing conclusions from the results given in these 2 tables, it should be remembered that the figures given are low, rather than maximum values, since in the majority of instances the basal value was not reached before the conclusion of the experiment . . ." (p. 343).

In observational periods extending over 6 to 10.5 hours post prandium, Murlin, Burton and Barrows ('36) report an average S.D.A. of fat in human subjects of 4.74% of the caloric content. For periods up to 7 hours post prandium, Krauss and Rettig ('29) secured an average S.D.A. for protein foods of about 32%, but neither the experiments of Abel ('43) nor those of Jahn and Strössenreuther ('28) indicate that the S.D.A. of proteins, or of high-protein foods or meals, terminates within a period of 6 or 8 hours. The common practice of deferring a determination of the basal metabolism of men and women until 12 or more hours have elapsed since the last meal, with the stipulation that the last meal should not be high in protein, carries the same implication.

Wachholder and Franz ('44) studied the S.D.A. of a mixed diet in a group of about 50 normal men; observations were made intermittently throughout the day, during which the usual 3 meals were served. The interval between breakfast and lunch was 5 hours, and that between lunch and supper, 5.5 hours. The total observed S.D.A. for the breakfasts, varying in size from 536 to 922 cal., averaged 6.6% of the calories consumed; that for the lunches, containing 812 to 1350 cal., averaged 9.5% of the consumed calories. It is not probable that the latter value includes all of the extra heat produced after lunch, but nevertheless the results show a distinct tendency for a greater proportion of the food calories to be dissipated as S.D.A., the larger the caloric content of the meal.

Post-prandial metabolism in the human is ordinarily observed by intermittent determinations of heat production following a meal of a definite description. During the period of observation, the subject must be inactive. To continue the observations until the S.D.A. of the experimental meal runs out may be extremely tiresome for the subject, and if accomplished may give metabolic measurements complicated by appreciable and variable activity. Wachholder and Franz ('44) took particular pains in their protracted experiments to make their subjects comfortable and to obviate this criticism; with what success we do not know. The purpose of the report to be made below is to present experimental data on the specific dynamic action of high-protein and high-carbohydrate meals in 2 groups of 6 adult men, and in particular to present a method of summing the extra calories post prandium over a reasonably long observational period of 6 to 7 hours and of estimating by extrapolation the total extra calories that would have been measured if the observations had extended over the entire period of elevated metabolism.

EXPERIMENTAL PROCEDURE

In connection with a comprehensive project involving the study of the effect of dietary modifications on the tolerance

of man to repeated exposure to intense cold, a series of tests were made on the 12 subjects of the experiment to measure the specific dynamic action (the heat increment) of the high-protein and the high-carbohydrate diets used in the first year's work. These tests are referred to by Keeton and associates ('46). The following quotation from this publication briefly describes the S.D.A. experiments:

"The heat increment of the experimental diets was determined by following the heat production of the subjects in the respective diet groups for a period of six hours after consumption of the 'A' meal containing approximately 1000 calories. The experiments were carried out with the subjects sitting quietly in a chair, under comfortable conditions (77°F.), and again under cool conditions (60°F.). In both series of tests the men were clothed only in light union suits, 90% cotton. The respired air was collected intermittently, every half hour for the first 2 hours post prandium, and then every hour."

For each S.D.A. measurement, the fasting metabolism of the subject, at least 12 hours after the last meal, was determined while sitting quietly in a chair. The heat production in all tests was estimated from the oxygen consumption and the total respiratory quotient, neglecting protein metabolism. The error made by this neglect is usually less than + 2% (Mitchell and Haines, '27), and under extreme conditions would not exceed + 6.58%. It is well known that the computation of non-protein respiratory quotients, for short periods of time and during variable rates of protein metabolism, on the basis of the nitrogen excretion in the urine, cannot be done with any degree of accuracy.

There were 12 subjects, men varying in age from 23 to 25 years. The subjects are described individually and in detail by Keeton et al. ('46, table 1). Their average weight was 69.7 kg and their average body surface area was 1.865 m² during the time the S.D.A. tests were carried out.

EXPERIMENTAL RESULTS

A description of the average experimental meals is given in table 1. These values were calculated from the amounts of the 2 diets consumed and the average composition of such foods taken from Bowes and Church ('42), supplemented by such direct analyses as were necessary. As regards the total day's food, these estimates were checked occasionally by the chemical analysis of composite samples of food, each taken

TABLE 1
Description of average test meals

DIET	ENERGY VALUE		DISTRIBUTION OF CALORIES		
	Total	Per m ² body surface	Protein	Fat	Carbo-hydrates
	cal.	cal.	%	%	%
High-protein	903	532	37	46	17
High-carbohydrate	1070	574	7	39	54

TABLE 2
Typical test meals for 2 diet groups

HIGH-PROTEIN MEAL		HIGH-CARBOHYDRATE MEAL	
	gm		gm
Tomato juice	250	Grapefruit juice	180
Beef	200	Puffed wheat	12
Cottage cheese	125	Cream	50
Milk	50	Bread	60
Bread	20	Milk	325
Butter	15	Butter	25
2 eggs		Sugar	23
1 egg white		Jam	50

over a 10-day period. The test meals were not all the same, but their calculated contents of proteins, fats and carbohydrates were fairly well equalized. The mineral and vitamin contents of the day's food were shown to be adequate by direct analysis.

Typical test meals of the 2 diet groups are shown in table 2.

The test meals in both diet groups averaged about 1000 cal. in fuel value, with ranges from 826 to 1136 cal. for the high-

protein group, and from 787 to 1325 cal. for the high-carbohydrate group. There was no observable correlation between the caloric intakes and the cumulative S.D.A. over a period of 6.5 hours post prandium. This was also the experience of Murlin, Burton and Barrows ('36) in their studies with high-fat diets. Apparently other factors determining heat production, not under experimental control, obscured the correlation that undoubtedly exists between the caloric content of meals of similar character and their calorogenic response in the body.

The data for the individual metabolism tests are too numerous to present in this paper. They were averaged for the 2 diets and the 2 environments for the intervals 0.5, 1.0, 1.5, 2.0, 2.5, 3.5, 4.5, 5.5 and 6.5 hours post prandium. All tests

TABLE 3

The average fasting sitting metabolism of the 2-diet groups of six men each

ENVIRONMENT	HIGH-PROTEIN DIET		HIGH-CARBOHYDRATE DIET	
	Cal./hr./m ²	R.Q.	Cal./hr./m ²	R.Q.
Comfortable	35.3	0.760	35.3	0.793
Cool	35.1	0.757	37.8	0.776

included in these averages were carried out within 15 minutes of the times indicated. In thus pooling the data for the different subjects, total heat productions for the time interval since conclusion of the experimental meal were calculated and the heat increments computed by deducting the fasting metabolism for the same time interval. A few of the data secured could not well be included in the group averages, either because the caloric content of the test meal was too small or because the time of testing was more than 15 minutes from the times selected for grouping.

The average fasting sitting metabolic rates of the 2 groups of 6 men each in the 2 environments are summarized in table 3. The temperature of the room did not have an appreciable effect upon the metabolic rate of the men on the high-protein diet. For the other diet group, the average metabolic rate

was 37.8 cal. per m^2 in the cool room and 35.3 cal. per m^2 in the comfortable room. An analysis of the differences in metabolic rate for each of the 6 men when in the cool room and in the comfortable room revealed 2 exceptions for which the rate was higher in the comfortable room. For the entire group of 6 men the probability is 0.12 (Student, '25) that the average group difference might have been produced by a random combination of the uncontrolled factors in the experiment.

The average elevation of the fasting metabolism throughout the 6.5 hours of observation was somewhat higher for the subjects of both diet groups in the cool room than in the warm room, but the difference was neither great nor of statistical significance. For the high-protein group, the cumulative S.D.A. for 6.5 hrs. averaged only 2.3 cal. higher for the tests in the cool room, a 1.9% increase. For the high-carbohydrate group, the average cumulative elevation was 4.3 cal., a 5.3% increase. However, this increase was not exhibited by 2 of the 6 subjects in this group.

The irregularities in the data from subject to subject and the inappreciable effect of the experimental environments on the results secured, if any effect at all was manifested, justified pooling the measurements secured in the 2 rooms for each of the experimental diets. The average values obtained in this way are summarized in table 4.

For the high-protein meals, the cumulative S.D.A. over the period of observation averaged 131.1 cal.; for the high-carbohydrate meals, the average was 85.3 cal. The calorigenic response of the high-protein meals exceeded that of the high-carbohydrate meals from 1.5 hours post prandium to the termination of the tests. On the former meals, the respiratory quotients exhibited a slight rise in the first 2 hours to a high of 0.793, and then decreased continuously to 0.760. On the high-carbohydrate meals, the R.Q. rose to 0.902 in 0.5 hr. and then decreased rather regularly also to 0.760, when the measurements were discontinued.

The purpose of the statistical analysis of the above data is twofold: (a) to smooth out the raw data by fitting to them a

TABLE 4
The average results of the metabolism trials

HOURS POST PRANDIUM	HIGH-PROTEIN DIET				HIGH-CARBOHYDRATE DIET			
	Number of tests	Number of subjects	Average R.Q.	Cumulative S.D.A. ¹		Number of tests	Number of subjects	Average R.Q.
				Observed cal.	Calculated ² cal.			
0	14	6	0.757	0	18	6	0.776
0.5	2	2	0.804	10.2	5	5	0.802
1.0	17	6	0.784	19.1	17	6	0.880
1.5	18	6	0.793	32.0	18	6	0.864
2.0	17	6	0.791	44.4	41.3	17	6	0.850
2.5	18	6	0.782	57.1	56.8	14	5	0.837
3.5	18	6	0.776	80.9	82.5	14	5	0.817
4.5	18	6	0.770	102.4	102.5	14	5	0.785
5.5	14	6	0.770	118.7	118.1	12	6	0.795
6.5	13	6	0.760	131.1	130.3	11	5	0.760
								Observed
								cal.
								Cumulative S.D.A. ¹
								Calculated ²
								cal.

¹ Specific dynamic action expressed in cumulative extra calories above basal heat production.

² Predicted from equation 1.

³ Predicted from equation 2.

suitable mathematical equation, permitting interpolation for any time post prandium, and, by differentiation, estimates of rates of extra calorie production at any time; and (b) to arrive at some reasonable estimate of the total cumulative S.D.A. for each type of meal, approximating what would have been obtained had the observations been continued until the fasting sitting metabolic rate had again been observed. On plotting the cumulative S.D.A.'s given in table 4 against hours post prandium, a pattern of points was obtained exhibiting a sigmoid shape, similar to a growth curve, with the self-accelerating phase confined to the first 1.5 to 2.0 hours post prandium. The data collected beyond this time seemed to be well described by the equation for the law of diminishing returns, as used by Brody ('45, p. 502) in his growth studies. Fitting the equation to the data of the 2 diet groups by the graphic method of determining the constant A (see Brody, p. 512) and by the method of averages in determining the constants B and k, the following equations were obtained:

for the high-protein group

$$S_p = 173 - 217.3 \cdot e^{-0.25936t} \quad (1)$$

and for the high-carbohydrate group

$$S_c = 108 - 118.5 \cdot e^{-0.20376t} \quad (2)$$

in which S_p and S_c are the cumulative S.D.A.'s for the high-protein and high-carbohydrate meals, respectively, expressed in calories, at time t , expressed in hours post prandium, and e is the base of the natural system of logarithms.

In figure 1, the experimental observations are plotted together with the above equations. The fit of the equations to the respective sets of data is extremely good at 1.5 hours post prandium and beyond for the high-carbohydrate diet, and at about 2.0 hours and beyond for the high-protein diet (see table 4). The course of the data prior to these times is of the self-accelerating, rather than of the self-inhibiting, type as these qualifying terms are used by Brody.

In equations (1) and (2), the first constant terms on the right side of the equations, i.e., 173 and 108 cal., are the

asymptotes to their respective curves, the values the curves are approaching but will never reach in finite time. They are, therefore, somewhat higher than the total S.D.A.'s.

In applying these equations to mammalian growth, the constants signify the mature weights of the respective species at infinity. For practical purposes, Brody considers the

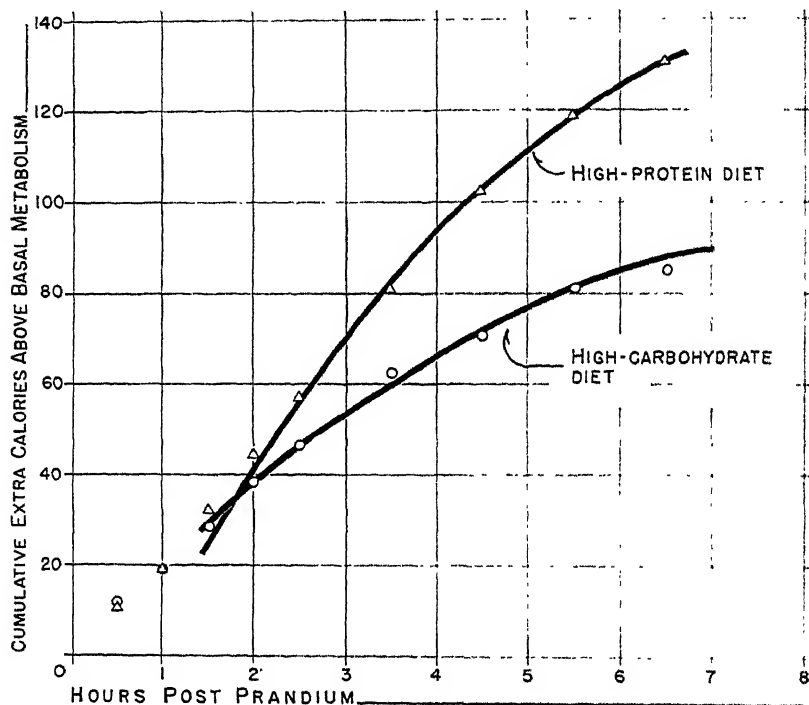


Fig.1 The increase in cumulative extra calories (S.D.A.) with time post prandium for high-protein and high-carbohydrate meals.

mature weight to equal 98% of the A constant. Applying the same conception to the S.D.A. equations, the total S.D.A. for the high-protein diet would be estimated at 170 cal. (0.98×173), a value that would be attained in 16.54 hours post prandium. For the high-carbohydrate diet, the estimate for total S.D.A. (108×0.98) is 106 cal. and would be attained in 15.12 hours post prandium. At the end of 12 hours post

prandium, the time ordinarily assumed as the length of the absorptive period in the human provided the last meal is not too high in protein, the cumulative S.D.A. for the high-protein diet is 162 cal., and for the high-carbohydrate diet, the value is 102 cal.

By differentiating equations (1) and (2), derived equations are obtained from which may be estimated the instantaneous rate of extra heat production at any time during the absorptive period. The differential equations are:

$$\frac{dS_p}{dt} = 54.35e^{-0.2501t} \quad (3)$$

for the high-protein diet, and

$$\frac{dS_c}{dt} = 31.25e^{-0.2677t} \quad (4)$$

for the high-carbohydrate diet. These equations are plotted in figure 2, starting with the time post prandium at which equations (1) and (2) describe the experimental data. The differentials $\frac{dS}{dt}$ will evidently be expressed in extra calories above the fasting sitting metabolism per hour.

The maximum S.D.A. for the high-protein diet is 33.0 cal. per hour at 2 hours post prandium, decreasing to 2.7 cal. per hour at the 12th hour post prandium, and to 1.0 cal. at the 16th hour. For the high-carbohydrate diet, the rate of extra caloric production is 21.0 cal. per hour at the peak 1.5 hours post prandium, decreasing to 1.3 at 12 hours and to 0.46 cal. at 16 hours.

The rate of extra caloric production is very small on both diets at 12 hours post prandium and quite insignificant at 16 hours. Taking the latter period for the high-protein diet, and the former for the high-carbohydrate diet as the period of active food absorption, we may estimate the *total* S.D.A. for the high-protein diet as 169 cal. and for the high-carbohydrate diet as 103 cal. Since the average caloric intake was 993 cal. on the high-protein diet, and 1070 cal. on the high-carbohydrate diet (see table 1), the total S.D.A. expressed in the usual terms is 17.0% of the ingested calories for the high-pro-

tein diet, and 9.6% for the high-carbohydrate diet. These values are higher than those of Benedict and Carpenter ('18), i.e., 12% and 6%, respectively, because they cover the entire absorptive period.

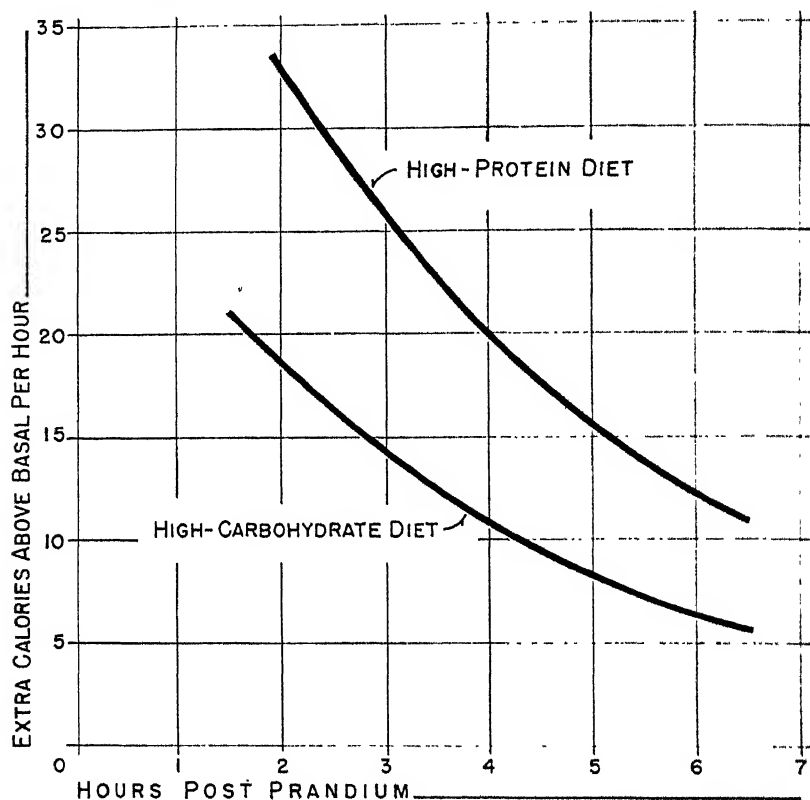


Fig. 2 The rate of extra calories production per hour after high-protein and high-carbohydrate meals.

The variation among individual subjects in the cumulative S.D.A. may be assessed from the total extra calories at the end of 6.5 hours post prandium. The coefficients of variation for the high-protein meals were 19% and for the carbohydrate meals, 16%, pooling in each case the results for the comfortable and the cool room. In the experiments of Wachholder and Franz ('44), the coefficients of variation in the total ob-

served S.D.A.'s for the different meals varied from 15 to 41%, averaging 28%.

DISCUSSION

The accumulation of extra calories above a base-line expenditure after a meal pursues a sigmoid course when plotted against time post prandium, i.e., the rate of accumulation increases for a period of 1.5 to 2.5 hours and then decreases until the metabolic stimulus, whatever it may be, is exhausted. The nature of the stimulus (or stimuli) is not known, but one may look at the situation from the standpoint of the plethora theory, championed and then abandoned by Lusk. Regardless of its validity, it does explain reasonably and more completely the observed facts concerning the specific dynamic action of nutrients and the factors affecting it than any other theory yet proposed.

According to the theory of plethora, the end products of digestion slowly establish a plethora in the tissues³ as long as the rate of inflow exceeds the rate of tissue utilization for purposes of oxidation or of synthesis. During this phase of the phenomenon, the establishment of a plethora, and consequently the S.D.A., proceeds at a self-accelerating rate. As the rate of inflow diminishes (and the rate of utilization possibly increases) the plethora subsides and the rate of S.D.A. decreases. That this decrease should follow the course of the law of diminishing returns is not surprising, since the slower and slower inflow of each successive unit of nutrients into the tissues would be expected to induce a smaller and smaller calorigenic response. The theory can accommodate the observed facts concerning the participation of the endocrine glands in the S.D.A. of food in so far as hor-

³ Arguments advanced against the theory too often assume that the plethora of nutrients in the tissues parallels that in the blood. When the rate of S.D.A. does not parallel the changes in nutrient concentration in the blood, the conclusion is drawn that the theory is invalid. However, there is no reason to believe that tissue concentration of nutrients and blood concentration run parallel in the post-absorptive period; for the amino acids, this has been known not to be true, since the classic studies of Van Slyke and Meyer ('13).

mones may modify the response of tissues to a calorigenic stimulus, i.e., their irritability (Mitchell, '27). Extrapolation of the curve expressing the relationship of cumulative S.D.A. and hours post prandium to the time of termination of the S.D.A. stimulus may be justified on the basis of the reasoning just discussed, as well as on the basis of the goodness of fit of the data to the mathematical expressions employed to describe them.

Above environmental temperatures setting the lower limits to the zone of thermic neutrality in the fasting man, the S.D.A. may reasonably be regarded as a wastage of food energy and even a burden upon the temperature-regulating mechanism. Below such temperatures it may serve a useful physiological purpose by sparing body tissues and increasing the tolerance to cold. But in either case it is a small item in the energy economy of man. After a high-protein meal containing approximately 1000 cal., the maximum rate of extra calorie production is 33 cal. per hour, decreasing in 5 and 6 hours, the usual period between daytime meals, to 15 and 12 cal. per hour, respectively (see fig. 2). After high-carbohydrate meals, the maximum rate is 21 cal. per hour, and the rates after 5 and 6 hours are 8 and 6 cal., respectively. Of the many human activities for which Sherman ('45, table 23) gives the calorie equivalent per hour, there is none, when allowance is made for basal heat expenditure, with as low a calorie equivalent as the S.D.A., even of a high-protein meal. Thus, the extra calories over basal for standing relaxed are 40 per hour; for hand sewing, 46 per hour; for dressing and undressing, 53 per hour; and for typewriting rapidly, 75 per hour.

The difference in S.D.A. between a high-protein and a low-protein meal of 1000 cal. is even less significant, starting at 12 cal. per hour at the peak, and diminishing to 8 and 6 cal. per hour, respectively, at 5 and 6 hours post prandium. It is not surprising, therefore, that Pitts, Consolazio and Johnson ('44) were unable to detect any appreciable increase in the thermal load of men working in a hot environment induced by an intake of 150 gm of protein daily, as compared with

an intake of 75 gm, the caloric intake remaining the same. Also, Keeton and coworkers ('46) found that the higher S.D.A. of a high-protein diet was not the determining factor in its effect upon tolerance to an intensely cold environment. Furthermore, Robinson and Lee ('47), working with domestic animals, did not secure any evidence that a high proportion of protein in the ration had any significant effect upon the reactions of the animals to heat.

SUMMARY AND CONCLUSIONS

The specific dynamic action of 2 diets containing 7 and 37% of protein calories was measured for 6 young men in each case, for periods of 6 to 7 hours post prandium, both in a comfortable and in a cool room. The rate of accumulation of extra heat above the fasting sitting metabolism after meals containing about 1000 cal. was found to follow a sigmoid course, the accelerating phase terminating at about 1.5 hours post prandium for the high-carbohydrate (low-protein) meals, and at about 2 to 2.5 hours post prandium for the high-protein meals. Thereafter, the rate of accumulation of extra calories followed the equation expressing the law of diminishing returns.

From equations of the latter type fitted to the experimental data, pooled for the 2 environments, the total S.D.A. was calculated, the results being 169 cal. for the high-protein meals and 103 cal. for the high-carbohydrate meals, or 17.0 and 9.6%, respectively, of the total calories consumed.

From these equations the hourly rate of extra calorie production was estimated on the basis of the corresponding differential equations. Comparing these rates with even the lightest types of muscular activity of men, it is evident that the S.D.A. of food is an inconsiderable item in the energy metabolism of active men, and in particular that high-protein meals as compared with low-protein meals exert an inappreciable effect in enhancing the thermal load of men working in a hot environment, or in protecting men against a cold environment.

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THE NATURE OF THE SUPPLEMENTARY VALUE OF THE PROTEINS IN MILLED CORN MEAL AND MILLED WHEAT FLOUR WITH DRIED FOOD YEASTS ¹

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In recent publications (Sure, '46, '47) it was reported that when 1, 3 and 5% of milled enriched flour or milled white corn meal ² are replaced by equivalent amounts of dried brewers' yeast (K) or a primary-grown cultured yeast (G)³, there resulted large gains of body weight and marked increases in protein efficiency ratios, as evident from gains in weight per gram of protein intake. Since Mitchell and Smuts ('32) found that the proteins of wheat are deficient in lysine and the proteins of corn are deficient in lysine and tryptophane, it was anticipated that the dried food yeasts contributed these amino acids in the protein supplementation of these cereal grains. However, part of the marked responses to the administration of the yeasts may have been due to the influence of other dietary essentials. In this investigation an attempt was made to determine to what extent lysine could supplement for growth the proteins in milled enriched wheat

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² This was a "white table corn meal," purchased in local grocery stores.

³ Furnished by Anheuser-Busch, St. Louis, Mo.

flour, and lysine and tryptophane the proteins in milled corn meal, as compared with different amounts of cultured yeast (G) added to the rations. All the experimental animals were kept on the same protein level. However, the rats which had supplementary administrations of lysine and/or tryptophane received additional small amounts of nitrogen furnished by these amino acids.

The terms "proteins in wheat" and "proteins in corn meal" are used here to mean the total proteins as they exist in the natural state in these cereal grains. The total protein was calculated from the nitrogen content found by analyses.

This study was conducted on Wistar strain albino rats, according to a technique recently described (Sure, '46, '47). The basal rations contained 89% wheat flour or 89% corn meal as sources of proteins, 2% cellu flour for roughage, and 4% of Sure's salt mixture no. 1 (Sure, '41). When a certain proportion of yeast was added to the rations, enough wheat flour or corn meal was removed to keep the protein content the same. Two per cent cod liver oil and 3% wheat germ oil in the rations furnished the fat-soluble vitamins. The following components of the vitamin B complex were administered to each animal daily separately from the rations: 100 μ g thiamine, 100 μ g riboflavin, 100 μ g pyridoxine, 100 μ g nicotinic acid, 600 μ g calcium pantothenate, 12 mg choline chloride, 12 mg p-aminobenzoic acid, and 3 mg inositol. The results of this study are summarized in table 1.

The work with the wheat flour was carried out for a period of 10 weeks and it was intended to conduct the experiments on the corn meal rations for an equal period. However, the rats on the unsupplemented corn meal rations began to fail completely after 6 weeks; hence, it was necessary to terminate this study earlier. The animals on the corn meal rations were, therefore, kept on experiments for only 6 weeks.

In table 1 are shown the results of additions of 1, 3 and 5% cultured yeast (G) to the enriched wheat flour rations. The animals on rations containing 89% enriched wheat flour served as the controls. The latter ration was also supple-

TABLE 1

Influence on growth¹ and protein utilization of additions of lysine or tryptophane, or both, and cultured food yeast (G) to the proteins in enriched flour and in milled corn meal. All data are averages

TYPE OF RATION	PER CENT IN RATION	NUMBER OF ANIMALS	DAILY INTAKE				GAIN BODY WEIGHT	IN- CREASE IN BODY WEIGHT	TOTAL FOOD INTAKE	TOTAL PRO- TEIN INTAKE	PROTEIN EFFICI- ENCY RATIO ²	INCREASE %
			Lysine	Trypto- phane	Lysine	Trypto- phane						
			mg	mg	mg	mg	gm	%	gm	gm		%
Enriched flour	89.0	30	28.1	.	348	31.9	0.88	..
Enriched flour Cultured yeast (G)	85.2 { 1.0 }	24	.	.	1.0	.	39.2	39.5	385	35.3	1.11	26.1
Enriched flour Cultured yeast (G)	77.7 { 3.0 }	24	.	.	3.1	.	51.0	81.5	385	35.3	1.44	65.6
Enriched flour Cultured yeast (G)	70.2 { 5.0 }	24	.	.	5.1	.	62.1	121.0	387	35.5	1.75	98.8
Enriched flour	89.0	24	8	.	.	.	43.5	54.8	389	35.7	1.22	38.6
Enriched flour	89.0	30	20	.	.	.	53.2	89.3	400	36.7	1.45	64.8
Enriched flour	89.0	24	40	.	.	.	60.4	114.9	411	37.7	1.60	81.8
Enriched flour	89.0	24	60	.	.	.	69.5	147.3	407	37.3	1.86	111.3
Corn meal	89.0	24	30.4	.	314	25.0	1.21	..
Corn meal Cultured yeast (G)	76.3 { 3.0 }	24	.	.	3.1	0.54	48.6	+60.0	315	25.1	1.93	59.5
Corn meal Cultured yeast (G)	67.9 { 5.0 }	24	.	.	5.1	0.90	60.6	+99.3	362	28.9	2.09	72.7
Corn meal	89.0	12	20	.	.	.	25.1	-17.4	272	21.7	1.16	..
Corn meal	89.0	12	10	.	.	.	33.5	+10.2	349	27.8	1.21	0.0
Corn meal	89.0	24	20	10	.	.	68.1	+124.0	407	32.4	2.10	73.5

¹ Changes in body weight.

² Gain in weight per gram of protein intake.

Note: The experimental period for the animals on the enriched flour rations was 10 weeks; for those on the corn meal rations, 6 weeks. The total protein content of each ration for the first groups of rats (flour rations) was 9.17%; for the second groups (corn meal rations) 7.97%.

mented daily with 10, 25, 50 and 75 mg lysine hydrochloride, which in terms of pure lysine, corresponded to 8, 20, 40 and 60 mg, respectively. These amounts of amino acids furnished each rat daily approximately 1.6, 4, 8 and 12 mg supplementary nitrogen, respectively.

It will be noted that optimum growth and greatest protein efficiency were obtained on the 60 mg lysine supplementation and on the 5% yeast addition to the ration. However, the enriched flour ration, supplemented with 20 mg lysine daily, produced gains in body weight and protein efficiency similar to those on the ration containing 3% of the G yeast. The latter ration, however, furnished only 3.1 mg lysine daily.⁴ It will also be noted that the enriched flour ration supplemented with 60 mg lysine produced a protein efficiency ratio of 1.86, which is very close to that produced by the 5% yeast-containing ration, which is 1.75; and the latter ration supplied only 5.1 mg lysine daily. The basal ration supplemented with 8 mg lysine daily produced a protein efficiency ratio of only 1.22. It would appear then that the additions of 3 and 5% of the G yeast, which supplied lower intakes than 8 mg lysine daily, stimulated growth by virtue of the addition of one or more other dietary essentials. One of these may be the amino acid, valine, since Light and Frey ('43) found that the proteins of wheat are deficient in valine as well as lysine. Another possible explanation is that the yeast may have furnished one or more of the as yet unidentified components of the vitamin B complex.

It is also apparent from table 1 that neither lysine nor tryptophane added alone improved the biological value of the proteins in milled corn meal. As a matter of fact, the addition of lysine alone reduced food consumption and lowered the protein efficiency ratio compared with results on the unsupplemented basal ration. However, the daily administration of 20 mg lysine and 10 mg tryptophane (which furnished about 5.5 mg of supplementary nitrogen) was accompanied by in-

⁴ The lysine and tryptophane contents of the G yeast were determined microbiologically at the laboratories of Anheuser-Busch, St. Louis, Mo.

creases in growth and protein efficiency similar to those characteristic of the ration containing 5% of the G yeast. The increased growth on the ration supplemented with these amino acids is due to increased food intakes. Since, however, the 5% yeast-containing ration supplied daily only 5.1 mg lysine and 0.9 mg tryptophane, the yeast must have furnished an additional dietary factor or factors to account for such gains in body weight with such low intakes of these amino acids.

SUMMARY

The increased biological value of the proteins in milled enriched wheat flour with dried food yeast (G) is due to the latter's provision of the amino acid, lysine, and also possibly to one or more other dietary essentials contained therein.

The increased biological value of the proteins in milled white corn meal with dried food yeast (G) is due to the latter's provision of the amino acids, lysine and tryptophane, and also possibly to one or more other dietary essentials contained therein.

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RELATIVE SUPPLEMENTARY VALUES OF DRIED FOOD YEASTS, SOYBEAN FLOUR, PEANUT MEAL, DRIED NON-FAT MILK SOLIDS, AND DRIED BUTTERMILK TO THE PROTEINS IN MILLED WHITE CORN MEAL AND MILLED ENRICHED WHEAT FLOUR ¹

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In recent studies (Sure, '46, '47) it was shown that when as little as 1 and 3% of dried brewers' yeast (K) or a dried cultured food yeast (G) or soybean flour replaced equivalent amounts of milled white corn meal or milled enriched wheat flour, there resulted pronounced increased growth and increases in protein efficiency ratios, as evident from gains in weight per gram of protein intake. Since the protein content of the yeasts was 45 to 50% and that of the soybean flour about 50%, the addition of even small amounts of these high-protein-containing foods produced appreciable increases in the total protein content of the rations. Therefore, the previous investigations dealt essentially with protein enrichment. In this investigation results are submitted on the relative

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supplementary values of several high-protein-containing foods to the total proteins as found in the natural state in milled white corn meal and in milled enriched wheat flour. No proteins were isolated from any of the foods used but their total protein content was calculated from nitrogen analyses.

Different batches of wheat flour, corn meal, non-fat milk solids and dried sweet-cream buttermilk varied in the total nitrogen content. However, in the construction of the rations, the necessary adjustments were made in the proportions of the high-protein-containing food supplements. In order to calculate the total protein, the conventional factor of 6.25 was not always used, but the different factors for various foods, as recommended by D. B. Jones ('31) were employed, i.e., 5.7 for wheat, 6.25 for corn, yeast, soybean flour, and 6.38 for the dried milk solids. Yeasts contain non-protein nitrogen, such as purines and pyrimidines. For this reason a minimum deduction of 10% was made in calculating the protein content of the yeasts.² Thus, the true protein content of the G and K yeasts used in this study is represented by 90% of nitrogen \times 6.25.

All the rations³ were so constructed that they contained the same total protein in the basal corn meal or wheat rations as those containing the various supplements. The amounts of soybean flour, peanut meal, dried non-fat milk solids, and dried sweet-cream buttermilk were added in such proportions that they furnished the same amounts of protein as those supplied by the G and K yeasts; and the total protein contents of all these rations were the same. Therefore, the results secured can be interpreted as true supplementary values.

² Personal communication from H. E. Carter, Department of Biochemistry, University of Illinois.

³ The dried food yeasts were furnished by Anheuser-Busch, St. Louis, Mo. The soybean flour, processed and solvent-extracted, was supplied by Archer-Daniels Company, Minneapolis, Minn. The peanut meal was a processed product, sold under the trade name of "peanut granules," and supplied by Trades Oil Mill Co., Fort Worth, Texas. The dried non-fat milk solids and dried sweet-cream buttermilk were spray-processed Kraft and Borden products.

This study was carried out on Wistar strain albino rats. The composition of the rations is given in a previous paper (Sure, '48). Each animal on the 89% corn meal and 89% enriched wheat flour rations had the following vitamin B complex daily supplement: 100 μ g thiamine, 100 μ g riboflavin, 100 μ g pyridoxine, 100 μ g nicotinic acid, 600 μ g calcium pantothenate, 12 mg choline chloride, 12 mg p-aminobenzoic acid, and 3 mg inositol. Each rat which had the 60% corn meal and the 60% wheat flour rations received the following daily vitamin B complex supplement: 50 μ g of each of the following: thiamine, riboflavin, pyridoxine, and nicotinic acid; 300 μ g of calcium pantothenate, 9 mg choline chloride, 6 mg p-aminobenzoic acid, and 3 mg inositol. The results of this investigation are submitted in tables 1 and 2.

From table 1 it is evident that on the ration containing 89% corn meal, the total protein content of which was 7.97%, peanut meal proved the poorest supplement. This may be due to low food consumption which may have been influenced by lack of palatability and/or amino acid deficiencies of the peanut meal, since amino acid deficiencies, like vitamin deficiencies, produce inanition (Rose and Epstein, '39). There seem to be no noteworthy differences between cultured yeast (G), soybean flour, dried non-fat milk solids and dried sweet-cream buttermilk when fed as protein supplements to a ration containing 75% milled white corn meal.

On the ration containing 60% corn meal, the protein content of which was only 6.08%, there undoubtedly existed pronounced amino acid deficiencies; hence, peanut meal must have furnished supplementary amino acids to corn meal proteins, because it produced a definite supplementary effect. Nevertheless, peanut meal proved the poorest supplement on the lower level as well as on the higher level of corn meal intake, compared with the food yeast, soybean flour, and dried milk solids. Here again, no marked differences in the relative supplementary values were found between the food yeast, soybean flour, dried non-fat milk solids, and dried sweet-cream buttermilk.

Relative supplementary values of cultured yeast (G), soybean flour, peanut meal, non-fat milk solids, and dried buttermilk, to the proteins in milled corn meal (average results per animal for a 6-week period)

TYPE OF RATION	CORN MEAL AND PROTEIN FOODS IN RATION	GAIN IN BODY WEIGHT	INCREASE IN BODY WEIGHT	TOTAL FOOD INTAKE	TOTAL PROTEIN INTAKE	PROTEIN EFFICIENCY RATIO ¹	INCREASE
	%	gm	%	gm	gm		%
<i>Group I</i>							
Corn meal	89.0	30.7	...	314	25.0	1.23	...
Corn meal	74.97 }						
Cultured yeast (G)	3.00 }	54.7	78.1	393	31.3	1.75	42.3
Corn meal	74.97 }						
Soybean flour	2.57 }	48.1	56.7	362	28.9	1.66	34.9
Corn meal	74.97 }						
Peanut meal	2.11 }	31.5	2.6	328	26.1	1.21	0.0
Corn meal	74.97 }						
Dried non-fat milk solids	3.56 }	47.8	55.7	366	29.1	1.64	33.2
Corn meal	74.97 }						
Dried buttermilk	3.70 }	50.0	62.8	369	29.3	1.70	39.0
<i>Group II</i>							
Corn meal	68.0	6.7	...	233	14.2	0.47	...
Corn meal	54.1 }						
Cultured yeast (G)	3.0 }	26.2	291	309	18.8	1.39	196
Corn meal	54.10 }						
Soybean flour	2.57 }	24.9	272	311	18.9	1.32	181
Corn meal	54.10 }						
Peanut meal	2.11 }	17.1	156	291	17.7	0.96	104
Corn meal	54.10 }						
Dried non-fat milk solids	3.56 }	23.8	255	269	16.4	1.45	208
Corn meal	54.10 }						
Dried buttermilk	3.70 }	22.9	242	262	15.9	1.44	206

¹ Gain in weight per gram of protein intake.

Note: There were 6 ♂ and 6 ♀ rats in each experiment in group I, and 12 ♂ and 12 ♀ rats in each experiment in group II. Every ration contained 7.97% protein in group I, and 6.08% protein in group II.

The results on relative supplementary values of dried food yeasts, soybean flour, peanut flour, and the dried milks to the proteins in milled enriched wheat flour are summarized in table 2. Enriched wheat flour was fed at 2 levels of intake with 2 and 3% dried yeast (G) as a supplement and the rest of the high-protein-containing foods were fed in proportions which supplied the same amounts of protein as the yeast. Enriched wheat flour was also fed at a 60% plane of intake with 2% dried brewers' yeast (K) added as a basis of comparison.

From table 2 it would appear that the dried buttermilk was the best supplement to enriched flour fed at a 89% level and that the non-fat milk solids was a close second. However, on a higher plane of food yeast intake and corresponding higher intake of the rest of the food supplements, the greatest increase in body weight was obtained on the dried buttermilk because of the greatest food intake. On very similar food intakes, however, the animals on the soybean flour gained considerably more in body weight and showed a higher protein efficiency ratio than the rats on the peanut meal. The animals on the dried yeast (G) equalled in protein efficiency ratio those on the dried buttermilk. But the outstanding result indicated in table 2 is the superior supplementary value obtained with the dried buttermilk in comparison with the non-fat milk solids. This is even more accentuated on the 60% enriched wheat flour intake (groups II and III, table 2). The inferiority of peanut meal as a supplement compared with the other high-protein-containing foods is also quite evident.

When this study was completed in 1947 the interpretation was first made that dried buttermilk probably contains an essential dietary factor that is deficient in non-fat milk solids. However, on examining the container of the dried non-fat milk solids ⁴ it was found that it was purchased in 1946, while the dried sweet-cream buttermilk ⁵ was obtained in 1947. Con-

⁴ A Borden product.

⁵ A Kraft product supplied by the American Dry Milk Institute.

<i>Group III</i>							
Enriched flour	60.0	12 ²	16.1	...	508	31.4	0.51
Enriched flour	51.3	}	41.6	155.3	629	38.9	1.06
Brewers' yeast (K)	2.0						
Enriched flour	51.3	}	31.4	95.0	569	35.2	0.89
Soybean flour	1.8						
Enriched flour	51.30	}	20.3	26.1	504	31.1	0.65
Peanut meal	1.63						
Enriched flour	51.30	}	41.0	154.6	609	37.6	1.09
Dried non-fat milk solids ²	2.64						
Enriched flour	51.30	}	54.3	237.3	665	41.1	1.52
Dried buttermilk ⁴	2.60						
<i>Group IV</i>							
Enriched flour	89.0	24	44.7	.	555	49.8	0.82
Enriched flour	76.68	}	83.3	86.3	654	58.8	1.42
Dried non-fat milk solids ³	3.50						
Enriched flour	76.68	}	72.0	61.1	574	51.6	1.40
Dried buttermilk ²	3.99						
<i>Group V</i>							
Enriched flour	60.0	12	23.2	...	505	30.6	0.76
Enriched flour	50.84	}	54.6	135.4	645	39.1	1.39
Dried non-fat milk solids ⁵	2.60						
Enriched flour	50.84	}	40.7	74.9	599	36.3	1.12
Dried buttermilk ²	2.96						

¹ Gain in weight per gram of protein intake.

² One-half males and one-half females.

³ This was a Borden product supplied in 1946.

⁴ This was a Kraft product supplied in 1947 by the American Dry Milk Institute.

⁵ All the dried milks used by groups IV and V were 1947 Kraft products supplied by the American Dry Milk Institute.

Note: All the rations containing 89% enriched flour and those following in such groups (I, II, IV) contained 8.99% protein. The rations containing 60% enriched flour and those following in such groups (III, V) contained 6.06% protein.

sequently, it was decided to repeat the work on these dried milks with new 1947 products. This was particularly necessary, since Hodson and Krueger ('47) found that on storage non-fat milk solids lost arginine, histidine, methionine and tryptophane.

The findings with the 1947 products are summarized in table 2, under groups IV and V. This table shows that the animals on the 1947 dried non-fat milk solids consumed more food than the rats on the 1947 dried buttermilk and, therefore, gained more weight. On the lower level of enriched wheat flour intake there was a definite higher protein efficiency ratio in favor of the dried non-fat milk solids. On the other hand, on the higher level of enriched flour intake, the protein efficiency ratio was the same on the 2 kinds of dried milks. The different supplementary values secured with the 2 batches of these dried milk solids may be due to different temperatures of drying, since Fairbanks and Mitchell ('35) demonstrated that with higher temperatures of drying there may occur losses of cystine in dried milks. Certainly, there appears to be no superiority in the biological value of the proteins in 1947 processed dried sweet-cream buttermilk over that of 1947 processed dried non-fat milk solids, when used as supplements to the proteins in enriched wheat flour.

SUMMARY

When used as supplements to the proteins in milled white corn meal, there were found very little differences between dried food yeasts, soybean flour, non-fat milk solids, and dried sweet-cream buttermilk. Peanut meal proved to be the poorest supplement.

When used as supplements to the proteins in milled enriched wheat flour, cultured yeast (G) and brewers' yeast (K) proved to be superior supplements to soybean flour. Peanut meal was the poorest supplement. Results with dried non-fat milk solids and dried sweet-cream buttermilk were variable, perhaps due to having used products that may have been subjected to different temperatures of drying.

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MINERAL METABOLISM STUDIES IN DAIRY CATTLE

IV. EFFECTS OF MINERAL SUPPLEMENTATION OF THE PREPARTAL DIET UPON THE COMPOSITION OF THE BLOOD OF COWS AND THEIR CALVES AT PARTURITION ¹

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Much attention has recently been given to the importance both of the calf's nutrition during fetal life and of the composition of colostrum. In general, these studies have dealt with vitamin A (Thomas et al., '46; Spielman et al., '46a, '46b; Wise et al., '46a, '46b), vitamin D (Hibbs, '46; Eaton et al., '47a, '47b), and vitamin E (Parrish et al., '47a, '47b) in the prepartal diet and/or in the colostrum of the dam and the effects of various treatments upon the blood and liver picture of the dam and/or of the newborn calf. Other studies in this connection have been concerned with choline (Waugh et al., '47), riboflavin (Sutton and Kaeser, '47) and globulin (Hansen et al., '46; Hansen and Phillips, '47a, '47b). Luecke et al. ('47) have reported data on the levels of iron, copper, cobalt, carotene, vitamin A, riboflavin, pantothenic acid, nicotinic acid and thiamine in the colostrum of the bovine.

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Very few data are reported on the effects of minerals in the prepartal diet upon the blood picture of newborn calves. Several reports, however, show that the level of plasma calcium is higher in the calf at birth than in the dam (Meigs et al., '19; Robinson and Huffmann, '26; Theiler et al., '27; Godden and Allcroft, '32). Groenewald ('35), using a low-mineral prepartal diet, found a similar situation for calcium and other elements in the blood of the dam and her calf. Robinson and Huffman ('26) and Godden and Allcroft ('32) found greater concentrations of inorganic phosphorus in the calf than in the dam.

In view of the lack of data giving a broad blood picture, especially that of the same blood specimen, of the cow and her progeny at the time of parturition, it was the purpose of this investigation to study the effects of mineral supplementation upon a number of blood characteristics and constituents of the cow and her newborn calf, to determine whether sex influenced the blood picture of the newborn calf, and to study the effects of colostrum upon the composition of the blood of the newborn calf.

EXPERIMENTAL

Four groups of cows were fed the following mineral supplements in the concentrate feed during at least the last 2 months of gestation: group I, basal concentrate feed (commercial dry cow and fitting ration); group II, basal concentrate feed plus 2.5% of CaCO_3 (c.p.); group III, basal concentrate feed plus 3.0% of calcite flour⁴ and group IV, basal concentrate feed plus 3.0% of Mico.⁵ The average contents of calcium, phosphorus and manganese of these concentrate feeds on dry basis are shown in table 1. All cows received

⁴ Calcite flour has the following average percentage composition: calcium, 33; magnesium, 2.6; manganese, 0.02; iron, 0.20; copper, 0.002 and zinc, 0.02. Product of the Limestone Products Corporation of America, Newton, New Jersey.

⁵ Mico has the following average percentage composition: calcium, 33; magnesium, 2; manganese, 0.20; iron, 0.20; iodine, 0.045; copper, 0.025; zinc, 0.01 and cobalt, 0.002. Product of the Limestone Products Corporation of America, Newton, New Jersey.

the same hay and silage and pasture in season. The groups were composed of the following numbers of cows: group I, 12; group II, 9; group III, 14 and group IV, 14.

An attempt was made to obtain blood samples from both the cows and their calves immediately following parturition and prior to the calves' sucking. Most of the data were obtained on samples procured within about 1 hour subsequent to calving and before the calf had access to colostrum; in 7 cases, however, blood was not obtained until the calves were several hours old (estimated average age of 4.5 hours) and after they had had some colostrum. No colostrum-fed calves were over 8 hours old when bled.

TABLE 1

Average calcium, phosphorus and manganese contents of concentrate feeds

GROUP	Ca	P	Mn
	%	%	%
I	1.00	0.64	0.0063
II	1.87	0.64	0.0058
III	2.04	0.67	0.0068
IV	1.97	0.65	0.0112

The determinations made, and the particular chemical methods used to determine the levels of certain blood and plasma constituents are as follows: hemoglobin (Sanford, Sheard and Osterberg, '33); reduced, oxidized and total glutathione (Woodward and Fry, '32); calcium (Clark and Collip, '25); inorganic phosphorus (Fiske and Subbarow, '25); acid and alkaline phosphatase at pH 5.0 and 9.3, respectively (King and Armstrong,⁶ '34); total plasma proteins, globulin and albumin (Looney and Walsh, '39); and ascorbic acid (Mindlin and Butler, '38) with certain modifications. The red blood cell count and packed red cell volume (hematocrit) were determined according to standard procedures and the mean

⁶ Procedure modified by Wiese et al. ('39). Unit of phosphatase defined as 1 mg phenol liberated from $\text{Na}_2\text{C}_6\text{H}_5\text{P}^{\text{O}}_4$ substrate in 30 minutes at 37°C. by 100 ml plasma.

corpuseular hemoglobin and volume were derived by calculation.

RESULTS AND DISCUSSION

The mean levels of various blood and plasma constituents of the cows and their progeny are shown by groups in table 2 and are summarized independently of groups in table 5. Although similar levels of hemoglobin and oxidized glutathione were found in the blood of cows and their calves, the blood of calves contained a greater number and was composed of a greater proportion of erythrocytes than that of the dams. The mean corpuseular hemoglobin and volume were correspondingly lower in the calves. In these animals, the whole blood contained markedly higher concentrations of reduced and total glutathione, and the plasma contained higher levels of ascorbic acid, acid and alkaline phosphatase, calcium and inorganic phosphorus than in the dams. The plasma of the cows, however, contained significantly greater quantities of total proteins, globulin and albumin than that of the calves. Of the differences between the mean levels of constituents in the blood or plasma of the dams and their calves, only the acid phosphatase for the groups receiving calcium carbonate (group II) and calcite flour ⁷ (group III), the inorganic phosphorus for group II, and the packed red cell volume for group III lacked mathematical significance at the 5% level; however, these differences (table 5) were significant between all dams and calves, disregarding group.

The concentrations of various blood and plasma constituents of the cows and of their calves were so similar that it seemed that calcium (groups II and III) and calcium with trace elements (group IV) were without effect upon the constituents of blood studied. It would appear that concentrate feeds containing about 1% calcium are as effective in the maintenance of the plasma calcium of both the dam and the calf as those containing 2% calcium when fed with good quality roughage. In this regard, Bodansky and Duff ('41)

⁷ See footnote 4, page 76.

TABLE 2

Blood and plasma composition of dams and their newborn calves (mean \pm standard error of the mean)

CATEGORY OF INTEREST	GROUP I		GROUP II		GROUP III		GROUP IV	
	12 Dams	12 Calves	9 Dams	19 Calves	14 Dams	14 Calves	14 Dams	14 Calves
BLOOD								
Hemoglobin (gm %)	12.88 \pm 0.32	13.27 \pm 0.60	13.53 \pm 0.22	13.97 \pm 0.45	13.22 \pm 0.17	12.97 \pm 0.47	13.55 \pm 0.29	14.31 \pm 0.40
Red cells (millions/mm ³)	6.73 \pm 0.30	10.46 \pm 0.70	6.82 \pm 0.29	10.68 \pm 0.74	7.61 \pm 0.59	9.34 \pm 0.39	7.29 \pm 0.20	10.44 \pm 0.57
Hematocrit (%)	35.42 \pm 1.15	41.77 \pm 2.29	35.78 \pm 0.95	42.54 \pm 1.26	36.48 \pm 0.67	39.19 \pm 1.87	37.86 \pm 1.15	45.92 \pm 1.62
Mean corpuscular								
Hb (μ g)	19.17	12.69	19.84	13.08	17.37	13.89	18.59	13.71
Mean corpuscular volume								
(μ^3)	52.71	39.93	52.46	39.83	47.94	41.96	51.93	43.98
Reduced glutathione								
(mg %)	41.51 \pm 1.77	59.86 \pm 3.21	37.65 \pm 2.83	57.96 \pm 3.38	32.87 \pm 2.49	40.04 \pm 5.87	40.81 \pm 1.83	59.26 \pm 2.99
Oxidized glutathione								
(mg %)	5.72	5.61	7.36	9.30	9.47	7.06	5.17	7.46
Total glutathione (mg %)	47.23 \pm 2.03	65.47 \pm 3.87	45.01 \pm 4.16	67.26 \pm 3.59	42.34 \pm 2.81	56.10 \pm 4.55	45.98 \pm 1.93	66.72 \pm 2.28
PLASMA								
Calcium (mg %)	9.38 \pm 0.24	11.84 \pm 0.47	9.78 \pm 0.69	11.45 \pm 0.46	9.75 \pm 0.31	12.33 \pm 0.45	10.52 \pm 0.94	12.65 \pm 0.57
Inorganic phosphorus								
(mg %)	5.31 \pm 0.57	6.88 \pm 0.29	5.60 \pm 0.78	6.55 \pm 0.29	5.36 \pm 0.49	6.56 \pm 0.24	4.26 \pm 0.28	6.17 \pm 0.23
Phosphatase, acid								
(units/100 ml)	1.12 \pm 0.19	2.36 \pm 0.30	1.21 \pm 0.15	2.11 \pm 0.72	0.96 \pm 0.15	2.36 \pm 0.31	1.72 \pm 0.37	1.88 \pm 0.30
Phosphatase, alkaline								
(units/100 ml)	3.36 \pm 0.61	17.24 \pm 3.75	5.10 \pm 1.42	18.04 \pm 4.19	4.97 \pm 1.07	25.72 \pm 5.11	4.73 \pm 1.20	15.12 \pm 2.83
Total proteins (gm %)	7.25 \pm 0.27	3.23 \pm 0.16	6.78 \pm 0.37	3.20 \pm 0.43	7.01 \pm 0.18	3.91 \pm 0.40	7.06 \pm 0.29	3.21 \pm 0.18
Albumin (gm %)	3.73	1.69	3.85	1.68	4.16	1.84	4.12	1.87
Globulins (gm %)	3.52 \pm 0.45	1.54 \pm 0.19	2.93 \pm 0.37	1.52 \pm 0.36	2.85 \pm 0.18	2.07 \pm 0.32	2.94 \pm 0.25	1.34 \pm 0.06
Ascorbic acid (mg %)	0.83 \pm 0.08	0.64 \pm 0.08	0.22 \pm 0.04	0.48 \pm 0.05	0.28 \pm 0.05	0.59 \pm 0.06	0.28 \pm 0.05	0.50 \pm 0.07

demonstrated that normal fetal growth and storage of calcium and phosphorus occur in rats despite great differences in the maternal intake of these elements, and concluded that the ability of the fetus to store calcium and phosphorus depends upon the maintenance of suitable concentrations of these elements in the blood on the maternal side and, probably also, on the fetal side of the placental barrier. Our data, in which a consistently higher level of calcium was found in the newborn calf than in the dam (average difference in plasma concentration, 2.19 mg %) as shown in tables 2 and 5, are not in agreement with those reported by Green and Macaskill ('28), who concluded that there are no characteristic differences between the blood of the calf and that of the dam relative to calcium content. The differences found in our study, however, are similar to those reported by Bogert and Plass ('23) for this element in the blood of the human mother and fetus.

The outstanding group differences observed were the lower levels of reduced and total glutathione of group III cows and calves than those of the respective animals of any other group. Although the calcite flour in the prepartal diet of these cows seemed responsible, the physiological significance of this difference is not known. These differences, though, were mathematically significant only between group III (calcite flour⁸ supplemented) and groups I (basal diet) and IV (Mico⁹ supplemented) for reduced glutathione in the dams' blood, and between group III and group IV for total glutathione in calves' blood.

With respect to the other constituents studied, it would appear that the basal ration (group I), and the basal ration supplemented individually by CaCO_3 (c.p.) (group II), calcite flour⁸ (group III) and Mico⁹ (group IV) were equivalent. Since the blood composition was not appreciably affected by the supplements used in this study, the data for the animals of all groups were combined and are presented in table 5.

⁸ See footnote 4, page 76.

⁹ See footnote 5, page 76.

The summarized data (table 5) for all cows compare favorably with those reported by Braun ('46) for cows (1.5 to 13 years old) on pasture.

Since the blood picture of mature bulls and cows is known to vary in a characteristic manner, the data for the calves were grouped in table 3 both according to sex and to the supplement fed their dams. These figures would indicate that the levels of the various constituents of blood studied were not influenced by the sex of the newborn calf or by the mineral supplement in the prepartal diet.

Many recent researches have been directed at the nutritive value of colostrum and have shown various effects of this food upon the composition (particularly vitamin substances) of the blood of the calf. In view of this and of the blood changes known to occur in calves during the first week of life (Wise et al., '47), a comparison was made of the blood picture of 7 calves which had received colostrum before blood was obtained, and 43 calves from which blood was taken immediately after birth and before these calves had suckled. These data are summarized in table 4 by groups including both sexes. Since mineral supplementation did not perceptibly influence the blood composition, the average blood picture for all calves, regardless of group, is shown in table 5. The data demonstrating the effects of colostrum did not appear to be related to sex; therefore, separate data for bull and heifer calves are not shown. The plasma levels of total proteins and globulin in the calves having had access to colostrum were strikingly higher than those of calves which were bled prior to nursing. The average age of the calves receiving colostrum was approximately 4.5 hours when the blood was procured for analyses.

Howe ('21) reported that the serum of the newborn calf before it has nursed does not contain proteins precipitable by 17.4% anhydrous Na_2SO_4 (i.e., blood of the newborn calf does not contain euglobulin or pseudoglobulin I). Within a few hours after the calf had received colostrum, Howe ('21) found large amounts of protein (euglobulin precipitable by 13.5%

TABLE 3

Blood and plasma composition of newborn bull and heifer calves (mean \pm standard error of the mean)

CATEGORY OF INTEREST	GROUP I		GROUP II		GROUP III		GROUP IV	
	6 Bulls	6 Heifers	5 Bulls	5 Heifers	6 Bulls	8 Heifers	9 Bulls	5 Heifers
BLOOD								
Hemoglobin (gm %)	13.75 \pm 0.74	12.69 \pm 1.00	14.02 \pm 0.80	13.91 \pm 0.54	13.40 \pm 0.30	12.65 \pm 0.79	13.96 \pm 0.61	14.88 \pm 0.30
Red cells (millions/mm ³)	10.98 \pm 1.07	9.94 \pm 0.96	10.80 \pm 0.81	10.55 \pm 1.33	8.94 \pm 0.42	9.54 \pm 0.54	10.41 \pm 0.69	10.48 \pm 1.05
Hematocrit (%)	43.02 \pm 3.06	40.27 \pm 3.71	41.98 \pm 2.15	43.10 \pm 1.55	38.91 \pm 2.46	39.39 \pm 2.85	45.09 \pm 2.53	47.24 \pm 1.34
Mean corpuscular								
Hb (μ g)	12.52	12.77	12.98	13.18	14.99	13.26	13.41	14.20
Mean corpuscular volume (μ^3)	39.18	40.51	38.87	40.85	43.52	41.29	43.31	45.08
Reduced glutathione (mg %)	62.17 \pm 5.90	57.94 \pm 3.62	52.32 \pm 4.92	62.60 \pm 3.36	41.50 \pm 13.10	53.75 \pm 5.07	61.40 \pm 3.95	55.41 \pm 2.76
Oxidized glutathione (mg %)	4.54	6.50	8.22	9.04	8.20	7.15	5.82	10.41
Total glutathione (mg %)	66.71 \pm 6.57	64.44 \pm 5.05	60.54 \pm 5.37	72.64 \pm 3.77	49.70 \pm 8.25	60.90 \pm 4.80	67.22 \pm 3.43	65.82 \pm 2.13
PLASMA								
Calcium (mg %)	12.19 \pm 0.86	11.48 \pm 0.41	11.55 \pm 0.53	11.32 \pm 0.82	12.84 \pm 0.91	12.01 \pm 0.47	13.30 \pm 0.78	11.49 \pm 0.53
Inorganic phosphorus (mg %)	6.87 \pm 0.46	6.89 \pm 0.40	6.35 \pm 0.52	6.75 \pm 0.41	6.49 \pm 0.32	6.62 \pm 0.36	6.25 \pm 0.29	6.03 \pm 0.41
Phosphatase, acid (units/100 ml)	2.00 \pm 0.27	2.60 \pm 0.46	2.35 \pm 0.40	1.87 \pm 0.21	2.78 \pm 0.62	2.09 \pm 0.33	2.07 \pm 0.40	1.57 \pm 0.46
Phosphatase, alkaline (units/100 ml)	18.31 \pm 5.64	16.53 \pm 5.40	9.36 \pm 2.00	24.99 \pm 5.79	29.94 \pm 7.82	23.71 \pm 7.06	17.80 \pm 3.19	10.84 \pm 6.14
Total proteins (gm %)	2.95 \pm 0.09	3.51 \pm 0.28	3.55 \pm 0.82	2.85 \pm 0.33	4.34 \pm 0.60	3.59 \pm 0.54	3.25 \pm 0.27	3.13 \pm 0.21
Albumin (gm %)	1.69	1.69	1.69	1.68	2.06	1.67	1.90	1.81
Globulins (gm %)	1.26 \pm 0.06	1.82 \pm 0.35	1.86 \pm 0.70	1.17 \pm 0.16	2.28 \pm 0.38	1.92 \pm 0.50	1.35 \pm 0.09	1.32 \pm 0.11
Ascorbic acid (mg %)	0.72 \pm 0.13	0.56 \pm 0.13	0.54 \pm 0.07	0.42 \pm 0.07	0.59 \pm 0.13	0.60 \pm 0.06	0.50 \pm 0.11	0.51 \pm 0.05

TABLE 4

Blood and plasma composition of newborn calves of both sexes as affected by colostrum feeding (mean \pm standard error of the mean)

CATEGORY OF INTEREST	GROUP I RECEIVING		GROUP II RECEIVING		GROUP III RECEIVING		GROUP IV RECEIVING	
	Colostrum		No colostrum		Colostrum		No colostrum	
	2 Calves	10 Calves	1 Calf	9 Calves	4 Calves	10 Calves	No calves	14 Calves
Homoglobin (gm %)	12.10 \pm 0.12	13.53 \pm 0.71	12.74	14.11 \pm 0.48	11.97 \pm 1.21	13.77 \pm 0.43		14.31 \pm 0.40
Red cells (millions/mm ³)	9.33 \pm 0.87	10.74 \pm 0.84	8.59	10.91 \pm 0.78	8.80 \pm 0.52	9.61 \pm 0.51		10.44 \pm 0.57
Hematocrit (%)	36.71 \pm 1.31	42.90 \pm 2.06	38.51	42.99 \pm 1.32	33.79 \pm 3.45	41.35 \pm 1.92		45.92 \pm 1.62
Mean corpuscular Hb (μ g)	12.97	12.60	14.83	12.69	13.60	14.33		13.71
Mean corpuscular volume (μ^3)	39.95	39.94	44.83	30.40	38.40	43.03		43.98
Reduced glutathione (mg %)	58.82 \pm 10.26	60.09 \pm 3.59	57.36	58.03 \pm 3.78	39.86 \pm 9.62	53.12 \pm 7.20		59.26 \pm 2.99
Oxidized glutathione (mg %)	7.92	5.10	3.68	10.01	6.20	6.99		7.46
Total glutathione (mg %)	66.74 \pm 14.56	65.19 \pm 4.11	61.04	68.04 \pm 4.04	46.06 \pm 7.39	60.11 \pm 5.31		66.72 \pm 2.28
Calcium (mg %)	11.83 \pm 0.37	11.84 \pm 0.56	10.73	11.51 \pm 0.51	11.63 \pm 1.03	12.64 \pm 0.46		12.65 \pm 0.57
Inorganic phosphorus (mg %)	7.04 \pm 0.19	6.85 \pm 0.82	5.90	6.62 \pm 0.32	7.03 \pm 0.48	6.38 \pm 0.27		6.17 \pm 0.23
Phosphatase, acid (units/100 ml)	3.36 \pm 0.98	2.11 \pm 0.26	3.15	1.99 \pm 0.22	2.68 \pm 0.56	2.21 \pm 0.39		1.88 \pm 0.30
Phosphatase, alkaline (units/100 ml)	12.21 \pm 7.70	18.50 \pm 4.40	12.60	18.72 \pm 4.68	35.77 \pm 11.69	22.37 \pm 5.56		15.12 \pm 2.83
Total proteins (gm %)	4.21 \pm 0.12	3.03 \pm 0.11	6.67	2.81 \pm 0.22	5.68 \pm 0.84	3.21 \pm 0.20		3.21 \pm 0.18
Albumin (gm %)	1.47	1.73	2.03	1.64	2.16	1.73		1.87
Globulins (gm %)	2.74 \pm 0.68	1.30 \pm 0.06	4.64	1.17 \pm 0.33	3.52 \pm 0.60	1.49 \pm 0.17		1.34 \pm 0.06
Ascorbic acid (mg %)	0.57 \pm 0.05	0.66 \pm 0.10	.46	0.48 \pm 0.20	0.62 \pm 0.18	0.58 \pm 0.06		0.50 \pm 0.07

Na_2SO_4 and pseudoglobulin I, precipitable by 17.5% Na_2SO_4). Ragsdale and Brody ('23) reported that neither globulin nor immune bodies are found in the newborn calf or in the newborn calf after the ingestion of normal milk; but, following the ingestion of colostrum, both are found in the blood serum. These investigators believed that globulin and immune bodies from the blood of the dam pass unchanged into the blood of the newborn

TABLE 5

Blood and plasma composition of cows and newborn calves immediately following parturition

CATEGORY OF INTEREST	ALL COWS (49) ¹	ALL CALVES (50) ¹	CALVES (43) ¹ NO COLOSTRUM ²	CALVES (7) ¹ COLOSTRUM ³
Whole blood				
Hb (gm %)	13.29	13.61	13.87	12.12
R.B.C.C. (millions/mm ³)	7.16	10.19	10.42	8.92
R.B.C.V. (packed cells) (%)	36.48	42.30	43.50	35.29
Mean corpuscular Hb (μmg)	18.56	13.36	13.31	13.59
Mean corpuscular volume (μ^3)	50.95	41.51	41.75	39.56
Reduced glutathione (mg %)	38.29	56.36	57.82	47.78
Oxidized glutathione (mg %)	6.79	7.08	7.21	6.33
Total glutathione (mg %)	45.08	63.44	65.03	54.11
Blood plasma				
Ca (mg %)	9.93	12.12	12.21	11.55
Inorganic phosphorus (mg %)	5.08	6.53	6.47	6.87
Phosphatase, acid (units/100 ml)	1.23	2.17	2.03	2.94
Phosphatase, alkaline (units/100 ml)	4.57	19.09	18.31	24.05
Total proteins (gm %)	7.04	3.41	3.08	5.40
Albumin (gm %)	3.99	1.78	1.75	1.94
Globulins (gm %)	3.05	1.63	1.33	3.46
Ascorbic acid (mg %)	0.28	0.56	0.55	0.58

¹ Numbers in parentheses indicate number of animals in each group.

² Calves receiving no colostrum before blood was procured for analysis.

³ Calves bled after colostrum ingestion and at an estimated average age of 4.5 hours.

calf through the mammary gland of the dam and through the alimentary tract of the calf. Ledingham ('07) showed that during infectious diseases when the immune body content of the blood increases, there is a corresponding rise in the blood globulin level (the euglobulin fraction showing greater change than the pseudoglobulin fraction). According to Little and Orcutt ('22), calves not fed colostrum at birth are without agglutinins, but agglutinins appear in the blood within 1 hour and reach a maximum within 5 hours subsequent to the ingestion of colostrum. This would suggest a rapid rate of absorption. The study of Ragsdale and Brody ('23) indicated that colostrum contains 6-12% globulin. Crowther and Rais-trick ('16) suggested that serum globulin is probably identical with colostrum globulin, although Smith ('46), using more refined techniques, has pointed out that the 2 globulins are not exactly identical. Recently Hansen et al. ('46) found the gamma globulin component of calves' serum to be extremely low at birth, but within 18 hours after the ingestion of colostrum the gamma globulin fraction was increased to about 10% of the serum proteins.

A tendency towards a lower level of hemoglobin and a lower erythrocyte count and red blood cell volume was observed in calves having ingested colostrum than in those not having had this food (tables 4 and 5). These observations may have been the result of an increased blood dilution in the calves which had access to colostrum. Since blood was obtained from these calves when their average age was approximately 4.5 hours, it would appear that colostrum globulin reaches the blood stream soon after ingestion. Perhaps an increased blood volume (or dilution) is a sequel to the increased plasma protein concentration. Contrary to the results of some of the early studies, the data shown in tables 4 and 5 would indicate the presence of small quantities of globulins [precipitable by half-saturated $(\text{NH}_4)_2\text{SO}_4$] in the newborn calf prior to the ingestion of colostrum.

SUMMARY

A study was made of the effects of calcium and of calcium with various trace elements in the prepartal diet upon the composition of the blood of 50 calves and their 49 dams immediately following parturition.

Mineral supplements fed for at least 2 months prepartum did not elicit perceptible effects upon the levels of calcium and inorganic phosphorus in the plasma of the newborn calves or of their dams. Of a number of whole blood and plasma constituents studied, the outstanding group differences found were the lower levels of reduced and total glutathione in both the cows and calves of group III (calcite flour¹⁰ supplemented) than in those of the other groups (basal diet alone, calcium carbonate supplemented, Mico¹¹ supplemented).

Greater concentrations of reduced and total glutathione, calcium, inorganic phosphorus, acid and alkaline phosphatase, and ascorbic acid and a greater number of erythrocytes were found in the blood of newborn calves than in that of their dams; a greater corpuscular hemoglobin content, corpuscular volume, and a higher plasma level of total proteins, globulin and albumin were found in cows than in their calves immediately following parturition.

Similar concentrations of the various blood constituents studied were found, regardless of the sex of the newborn calf.

Seven calves which received colostrum prior to the procurement of blood for analyses at an average age of 4.5 hours had strikingly higher plasma levels of total protein, globulin and albumin than those which had not been nursed by their dams. The blood of the calves which had ingested colostrum contained slightly less hemoglobin, smaller numbers of erythrocytes and a smaller proportion of the whole blood volume consisting of erythrocytes than the other calves, which may have been due to dilution of the blood. Small quantities of globulin [precipitable by half-saturated $(\text{NH}_4)_2\text{SO}_4$] were

¹⁰ See footnote 4, page 76.

¹¹ See footnote 5, page 76.

demonstrable in the plasma of newborn calves prior to the ingestion of colostrum.

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SELF SELECTION OF DIET

VIII. APPETITE FOR FATS¹

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TWO FIGURES

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Deuel and Movitt ('44) reported that rats preferred diets containing butterfat to those containing corn oil, cottonseed oil, olive oil, peanut oil, soybean oil, or margarine. This preference was ascribed to the flavor of the butter. Parrish, Shimer and Hughes ('46), using the initial preference method of determining food choice, could find little evidence of a preference by rats for foods containing butterfat to those containing corn oil. It was shown in this laboratory that when rats were offered their choice of sucrose, hydrogenated vegetable oil, casein, and a salt mixture, a majority of animals selected fat to supply the largest part of their caloric requirement (Scott, '46).

The present report describes experiments in which other fats (corn oil, cottonseed oil, and butterfat) were used as choices in place of or in addition to the hydrogenated fat, as well as a selection experiment in which the fats were incorporated in mixed diets. This series completes a group of experiments in which proteins other than casein (Scott and Quint, '46) and carbohydrates other than sucrose (Scott and Verney, '47) were studied.

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EXPERIMENTAL

In each of the first 3 experiments, 10 male and 10 female weanling rats were placed in individual cages and allowed to eat a mixed diet² offered in all of 4 cups. The experiments differed in that the sources of fats in the diets were different. Corn oil was used in the first experiment, alkali-refined cottonseed oil in the second, and butterfat in the third.³ Vitamins were fed separately as pills.⁴ During a 3-week control period, the amount eaten from each cup was recorded daily and the cups were then interchanged in a predetermined random manner. In the 3-week experimental period that followed, the choices given the animals were sucrose, casein, salt mixture, and the same fat as in the control period, each in a separate cup. The amount eaten of each was recorded and the cups interchanged daily as before.

In the fourth experiment, 20 male and 20 female weanling rats were divided into 4 groups such that each animal had 1 littermate of the same sex in each of the other 3 groups. The members of each group were fed one of the following mixed diets in all of 4 cups during the control period: (1) 10% hydrogenated vegetable oil⁵ diet; (2) 10% corn oil diet; (3) 10% cottonseed oil diet; (4) 10% butterfat diet; with the other constituents the same as in the standard diet. In the experimental period all animals were given their choice of these 4 mixed diets in separate cups.

In the fifth experiment, each of 20 rats was placed in a cage with 6 cups, all of which contained a diet consisting of 24% casein, 62% sucrose, 4% salt mixture, and 2.5% each of hy-

² The mixed diet consisted of 62% sucrose, 10% fat, 4% salt mixture (Jones and Foster, '42) and 24% casein (Labeo "vitamin-free").

³ Corn oil was obtained from Corn Products Refining Company, cottonseed oil from Interstate Cotton Oil Refining Company, and butterfat by washing and drying a commercial sweet butter.

⁴ One pill was given each rat daily. It contained approximately 60 μ g thiamine hydrochloride, 120 μ g riboflavin, 90 μ g pyridoxine hydrochloride, 150 μ g calcium pantothenate, 10 mg choline chloride, 1 mg α -tocopherol, and 55 I.U. vitamin A and 11 I.U. vitamin A as 0.001 ml Natola; all in a dextrin-powdered sugar base.

⁵ "Primex."

drogenated vegetable oil, corn oil, cottonseed oil, and butterfat. During the experimental period, 7 choices were allowed the animals in separate cups. These were sucrose, casein, hydrogenated oil, corn oil, cottonseed oil, butterfat, and salt mixture.

RESULTS

The growth and food consumption of the animals in the 3-week control period are shown in tables 1 and 2. In the

TABLE 1
Growth and food consumption in the control period¹

EXPERIMENT	FAT SOURCE	MALES		FEMALES	
		Wt. gain	Food eaten	Wt. gain	Food eaten
		<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
1	Corn oil	83.6 ± 4.0	161.4 ± 7.7	74.5 ± 2.1	154.7 ± 4.7
2	Cottonseed oil	78.4 ± 4.5	156.4 ± 9.5	62.6 ± 3.0	136.3 ± 6.9
3	Butterfat	81.8 ± 4.8	167.4 ± 9.3	66.8 ± 3.1	146.2 ± 6.6
5	Mixture	47.5 ± 7.7	126.3 ± 11.7	43.3 ± 4.7	127.2 ± 8.9

¹ All data in terms of mean and standard error of the mean.

TABLE 2
Growth and food consumption during the control period (experiment 4)

FAT SOURCE	WT. GAIN	FOOD EATEN
	<i>gm</i>	<i>gm</i>
Hydrogenated fat	73.7 ± 4.1	155.9 ± 6.3
Corn oil	77.4 ± 4.0	155.0 ± 4.9
Cottonseed oil	62.0 ± 2.9	140.3 ± 6.9
Butter	71.0 ± 4.4	152.3 ± 8.1

7-choice experiment, the animals grew rather poorly for no obvious reason. Otherwise, the nutritional value of the fats, except that of cottonseed oil, appeared to be approximately equivalent. This was indicated by data on weight gains in the littermate experiment shown in table 2.

The choices of the animals during the control period in the first 3 and fifth experiments are shown in figure 1, while the

choice of fats in the fifth experiment is shown in figure 2. The mixed diets selected by the animals in the fourth experiment are summarized in table 3.

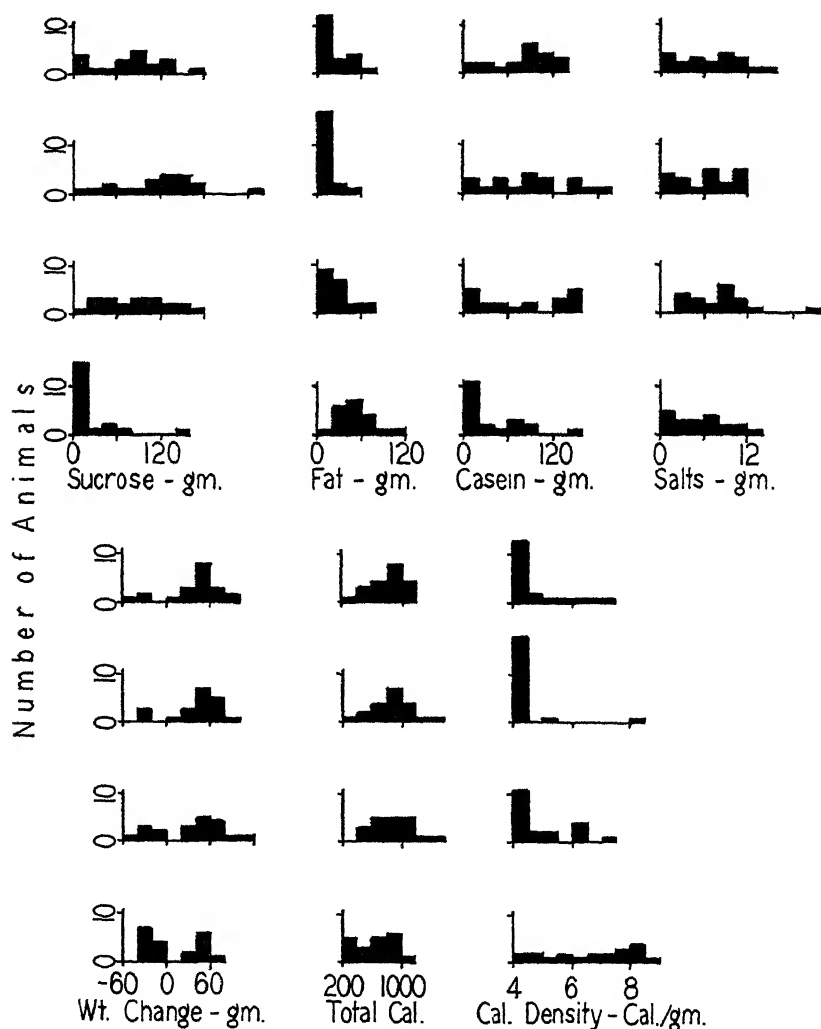


Fig. 1 Weight changes and food selections of rats during a 3-week period. The fat offered was in each case: Top histogram—corn oil; second histogram—cottonseed oil; third histogram—butterfat; bottom histogram—4 fats simultaneously.

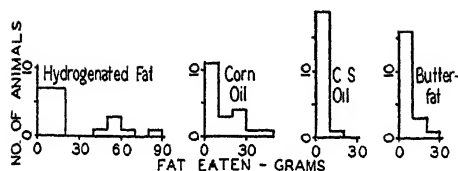


Fig. 2 Selection of fats when offered simultaneously.

DISCUSSION

In an earlier experiment (Scott, '46) where the choices offered were with respect to sucrose, salts, casein, and hydrogenated fat, 67% of the animals chose more fat in terms of calories than any other item, while 24% selected more sucrose, and 9% ate more protein. Hydrogenated fat thus being the food of choice, one might have expected more profound changes in the selection of other components if one offered a fat that was disliked than if one offered a protein or carbohydrate that was disliked.

In these experiments, none of the other fats was preferred as much as hydrogenated vegetable oil. The decreasing orders of preference were: 1 fat as choice, or fats incorporated in a diet — hydrogenated fat > butterfat > corn oil > cottonseed oil; 4 fats offered simultaneously — hydrogenated fat > corn oil > butterfat > cottonseed oil. These effects may have been due to the flavor of the fats and oils. Only the hydrogenated fat

TABLE 3

Appetite for mixed diets

DIETARY FAT SOURCE IN CONTROL PERIOD	WT. GAIN	FOOD EATEN	APPETITE FOR DIET CONTAINING ¹			
			Hydrogenated fat	Corn oil	Cottonseed oil	Butter
	gm	gm				
Hydrogenated fat	61.2 ± 7.7	226.1 ± 18.1	17.4 ± 9.2	-7.4 ± 3.7	-20.9 ± 3.0	11.2 ± 7.0
Corn oil	64.1 ± 7.6	235.5 ± 14.0	25.5 ± 8.7	-5.3 ± 6.0	-30.4 ± 7.1	9.7 ± 7.9
Cottonseed oil	75.0 ± 5.1	242.2 ± 7.7	24.5 ± 9.3	-6.5 ± 7.3	-13.6 ± 7.9	-4.8 ± 10.4
Butter	77.0 ± 7.6	247.2 ± 14.2	31.5 ± 13.4	-5.0 ± 9.0	-15.8 ± 3.0	-10.8 ± 7.7
Average	69.3 ± 3.5	237.7 ± 6.8	24.7 ± 5.0	-6.0 ± 3.3	-20.2 ± 2.9	1.4 ± 4.3

¹ Change in per cent eaten (experimental minus control) from corresponding cups during experimental and control periods. A positive change is indicative of a preference.

had an entirely bland flavor, and only this fat did not undergo flavor change on standing at room temperature. The cottonseed oil in particular became rancid quickly when incorporated in a diet. An attempt was made to keep fresh fats as choices. It was realized, however, that in any case the reaction of the animal to various degrees of rancidity or other flavor changes of the fats was not predictable.

The result of offering an unpopular fat such as corn or cottonseed oil as a choice was the selection by the animals of unusually large amounts of sucrose and casein. When several fats were offered as choices, little sucrose or casein was eaten.

These results and those obtained on other carbohydrates (Scott and Verney, '47) and on other proteins (Scott and Quint, '46) lead to 1 general conclusion: In this type of experiment, each food factor is selected or rejected on its own merits relative to the merits of the other possible choices. Accordingly, an item is selected by most animals if it is liked as well or better than the other possible choices and rejected by most animals if it is not liked as well. Such acceptance or rejection is generally not related to the nutritional nature of the choice, i.e., whether it is a carbohydrate, fat, or protein. One can increase the acceptability of a given choice by making other choices less acceptable or fewer in number, or decrease it by making them more acceptable or more numerous.

This interdependence of appetites is well illustrated in table 4, where data on acceptance from several experiments are compiled. Adopting a 20 to 1 level of significance, it will be noted that the only time less than the usual number of animals ate protein was when it was disliked (egg albumen); but more than the usual number ate it if several proteins were offered, or if the fat was unpopular (corn and cottonseed oils). Less than the usual number ate carbohydrate if it was disliked (lactose); but more ate it if it was well liked (starch), if more than 1 carbohydrate was offered, or if the fat offered was disliked (corn and cottonseed oils and butter). Less than the usual number selected fat if it was disliked (corn and cottonseed oils) or if several carbohydrates were offered.

There is little evidence in these studies that would indicate what properties of a given selected food item determine its acceptability for a rat. One could postulate that a pleasant flavor or texture, or the production of a subjective feeling of well-being would determine a favorable reaction, while an unpleasant flavor or texture, or unpleasant subjective symptoms, would have the opposite effect. In fact, there is no definite evidence in these studies that any factors other than

TABLE 4
Acceptance of various food choices by rats

CHOICES	NO. OF ANI- MALS	ANI- MALS SELECT- ING PRO- TEIN ¹	PROBA- BILITY ²	ANI- MALS SELECT- ING CARBO- HYDRATE ³	PROBA- BILITY ²	ANI- MALS SELECT- ING FAT ⁴	PROBA- BILITY ²
Casein, Sucrose, Primex ⁵	87	53	1.000	43	1.000	76	1.000
Sucrose, Primex ⁶ and							
Lactalbumin	20	11	.777	7	.263	19	.490
Fibrin	20	13	.408	12	.503	17	.727
Egg albumen	20	6	.006	6	.126	20	.145
Four proteins	30	27	.010	18	.501	30	.140
Casein, Primex ⁷ and							
Starch	20	15	.214	16	.012	16	.290
Lactose	20	13	.777	0	<.001	20	.145
Dextrin	20	15	.214	14	.126	17	.727
Four carbohydrates	20	13	.777	19	<.001	13	.009
Casein, Sucrose and							
Corn oil	20	18	.010	16	.012	12	.002
Cottonseed oil	20	17	.036	19	<.001	8	<.001
Butterfat	20	14	.509	19	<.001	19	.490
Four fats	20	9	.152	6	.126	20	.145

¹ Number of animals that ate enough protein to gain weight over a 3-week period.

² Probability that, solely as a result of random sampling, a distribution of animals into groups that did or did not eat a particular choice could be as improbable as that found, provided the expected distribution into groups was that observed in the casein, sucrose, Primex experiment.

³ Number of animals selecting at least 20 gm carbohydrate during a 3-week period.

⁴ Number of animals selecting at least 10 gm fat during a 3-week period.

⁵ Data taken from Scott ('46). Salt mixture was an additional choice in all cases.

⁶ Data from Scott and Quint ('46).

⁷ Data from Scott and Verney ('47).

the animals' subjective response to each group of choices are important in influencing selection. Because one cannot anticipate what the reaction of a given rat to a particular choice will be, the selections made under these conditions, while quite definite in some cases, often seem quite arbitrary. In consequence, the nature of selections from purified components of a diet did not appear to bear a demonstrable relation to the nutritional or physiologic requirements of the animals.

SUMMARY

Hydrogenated vegetable oil was more generally liked by young rats than butterfat or corn or cottonseed oils. When an unpopular fat was given as a choice, the rats selected much more casein and sucrose than when the choice was hydrogenated fat. It was concluded that choice of foods when components of a diet were offered was not related to the nutritional nature of the choices (i.e., whether a given choice was fat, carbohydrate, or protein), but was more probably dependent on the animals' subjective response to each particular choice.

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EFFECT OF PROTEIN LEVEL ON THE LYSINE REQUIREMENT OF THE CHICK

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FIVE FIGURES

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In the quantitative determination of the amino acid requirements of the chick, the level of protein has been kept at approximately 20% of the diet (Almquist, '47), but the effects of variation in the protein level are not known. An investigation of the problem should help in the exact definition of the amino acid requirements, and in the determination of the wisdom of feeding, at a higher dietary level, a protein slightly deficient in an essential amino acid in order to provide a satisfactory amount of the deficient amino acid in the diet.

It is well known that proteins slightly deficient in 1 amino acid can be fed in amounts which furnish more than the minimum level of protein, in order to satisfy the requirements for that particular amino acid. This is illustrated by the feeding of various levels of wheat gluten and other protein concentrates to rats (Barnes et al., '45), and by the feeding of 30% casein to satisfy the chick's arginine requirement, which is not met by 20% casein (Klose, Stokstad and Almquist, '38). Data are needed to determine the efficiency with which these high levels are used.

The lysine requirement was chosen because it has been well established by the use of several different proteins (Almquist, '47), and because there is a protein source available

(sesame meal) which, when fed at the 20% protein level, furnishes adequate amounts of all essential amino acids except lysine (Grau and Almquist, '44).

METHODS

White Leghorn chicks were reared on a stock diet for the first 2 weeks after hatching, then were segregated on the basis of weight into groups of 7 or 8 chicks, and given the experimental diets. The birds were housed in electrically heated battery brooders with wire floors; feed and water were supplied *ad libitum*. The chicks were weighed every other day during an 8-day period, and feed consumption was determined after 8 days. The basal diet consisted of calcium gluconate 8, tricalcium phosphate 2, sodium dihydrogen phosphate ¹ 1, potassium chloride 0.6, sodium chloride 0.5, choline chloride ² 0.2, inositol ³ 0.1, cholic acid ³ 0.1, crude soybean oil 5, mixed tocopherols ⁴ 0.05 and fortified sardine oil (3000A-400D per gm) 0.25 gm per 100 gm diet, and thiamine 1, riboflavin 1, pyridoxine 1, nicotinic acid 5, calcium (d) pantothenate 3, 2-methyl-1,4-naphthohydroquinone diacetate 1, biotin 0.1, pteroylglutamic acid ² 1, manganese 10, silicon 46, magnesium 48, aluminum 8, iron 14, copper 1, zinc 1, iodine 0.8 and cobalt 0.5 mg per 100 gm diet. Sesame meal and L-lysine monohydrochloride monohydrate from commercial sources were added as desired, and glucose ⁵ was added to a total of 100 gm. The sesame meal contained 43.6% crude protein ($N \times 6.25$).

¹ In some early experiments, dipotassium phosphate was used instead of sodium phosphate. In order to retain the same levels of potassium, phosphorus and sodium, changes were made in the levels of the chlorides.

² Provided by Lederle Laboratories Division, American Cyanamid Co., through the courtesy of Dr. T. H. Jukes.

³ Present only in the early experimental diets; omitted after it was found to have no effect on growth.

⁴ Concentrate of natural mixed tocopherols (15%), Distillation Products, Inc., Rochester, N. Y.

⁵ Cerelease.

The growth rates were obtained by multiplying the average gain per chick per day by 100 and dividing by the average weight during the experiment, to give the per cent gain per day (Grau and Almquist, '43).

RESULTS

In figure 1, the growth rates for the various groups are plotted against the levels of supplementary lysine, at 4 protein levels. When only 5% protein was present, the best growth obtained was only about 1% per day, and this rate

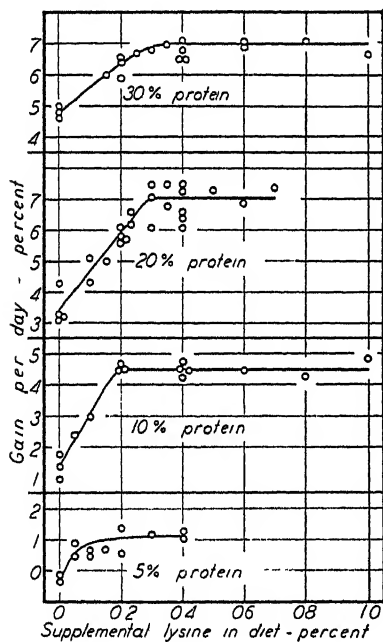


Figure 1

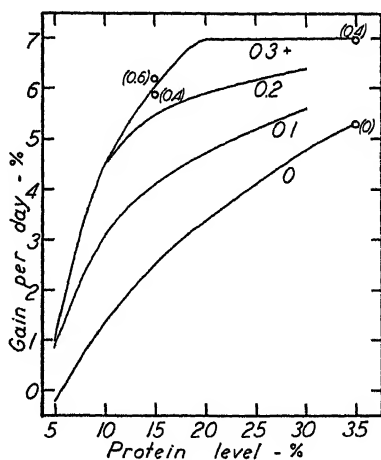


Figure 2

Fig. 1 Effects on growth of different levels of protein and of supplemental lysine. Each circle represents a group of 7 or 8 chicks, kept for 8 days on the diet after an initial period of 2 weeks on a control diet.

Fig. 2 Effects of protein level on growth rate, at various levels of supplemental lysine. The curves are drawn from the curves of figure 1. The circles represent additional experimental groups at protein levels other than those plotted in figure 1; their lysine levels are enclosed in parentheses. The other numbers are levels of supplemental lysine.

required the addition of approximately 0.1% lysine.^a Although the point of inflection of this curve is not sharply defined, the rapid initial rise indicates that the minimum amount of lysine for this level of protein is approximately 0.1%. It is apparent that higher levels had no significant effect on the growth rate. The minimum supplemental lysine level for optimum growth with 10% protein is sharply defined at 0.2%; the growth rate was not affected by the feeding of higher levels of lysine. The 20% protein curve is established by groups from 6 different experiments, including 1 experiment reported previously (Grau, Kratzer and Asmundson, '46). While there was more variation at this protein level, the averages at any one lysine level fall close to the line drawn. The curve indicates that 0.3% added lysine was needed for maximum growth, which was 7% per day, as compared with 4.5% per day with 10% protein. With 30% protein, a growth rate of 4.7% per day was realized without supplemental lysine; about 0.3% added lysine supported maximum growth.

In figure 2, these data are plotted in order to show the effect on growth of variation in the protein supply at several constant levels of supplemental lysine. The curves are taken from the curves of figure 1, except for 4 experimental points which are added from groups fed 15% and 35% protein. Not enough data for these protein levels were available to define curves for figure 1.

Even without supplemental lysine, the growth rate increased as the protein level increased, in a manner similar to that observed by Barnes et al. ('45). Addition of 0.1% lysine increased the growth rate at all protein levels, but 0.2% lysine was effective only at protein levels above 5%. Similarly, 0.3% lysine was effective only when the protein level was above 10%; curves for lysine levels above 0.3% coincided with that of 0.3%.

^a All data are calculated on the basis of lysine itself, although the monohydrochloride monohydrate was the form used.

Sesame meal protein contains 2.8% lysine (Block and Bolling, '45), so that the concentration of lysine in the basal diet is 0.14% at the 5% protein level; 0.28% at 10% protein; 0.56% at 20% protein; and 0.84% at 30% protein. When these basal levels are taken into account, the growth curves become oriented as shown in figure 3. These curves demonstrate clearly that when expressed in per cent of the diet needed to promote maximum growth, the lysine requirement increased

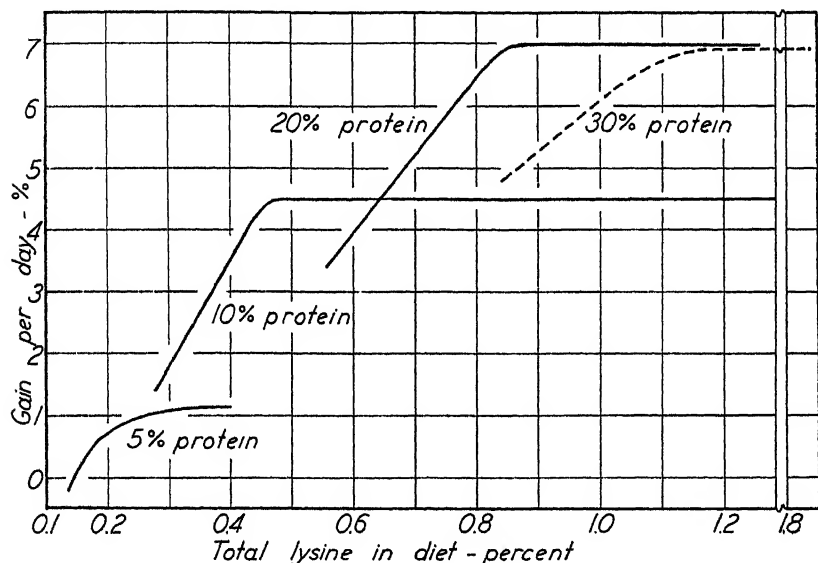


Fig. 3 Effects on growth of different levels of lysine and of protein. The curves are those of figure 1, with account taken of the lysine furnished by the sesame meal.

as the protein level increased, even at the 20% and 30% protein levels, where the maximum growth was also the best growth attainable by chicks under these conditions. The 20% protein level required 0.87% total lysine, which agrees remarkably well with the level (0.9%) originally set by Almquist and Mecchi ('42).

To ascertain whether or not the differences between protein levels were nullified by differences in feed consumption, actual

lysine intakes were calculated for each group. In figure 4, the results are plotted against the growth rate.⁷ It is apparent that at any given growth rate below the maximum, more lysine is ingested at high, than at low protein levels.

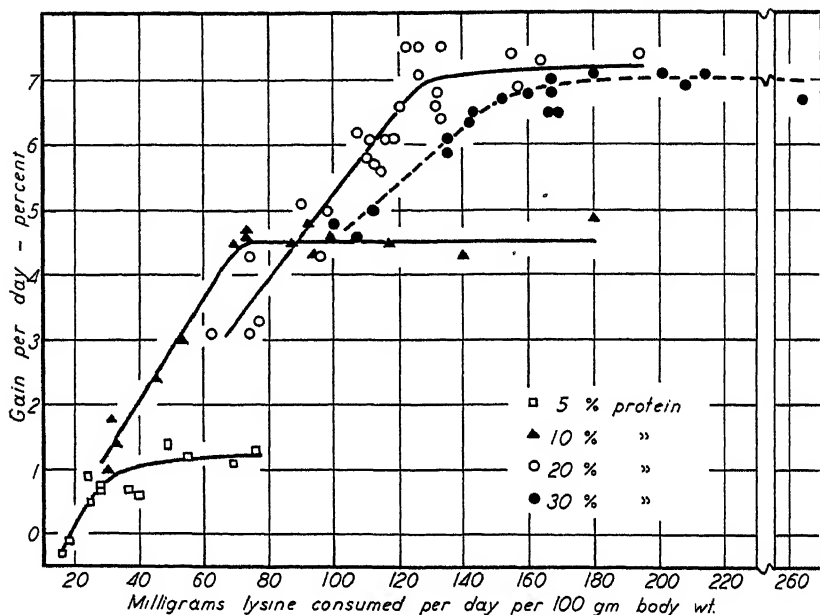


Fig. 4 Lysine consumption related to growth rate at 4 protein levels.

DISCUSSION

Since the conclusions drawn from this investigation are based primarily on growth rates of young animals, this basis for establishment of requirements should be justified. The strongest argument in favor of gain in weight as an adequate measure of protein and amino acid utilization is that there exists a constant tissue protein composition, regardless of the dietary level of an essential amino acid (Grau, '47). The question which remains is whether or not body weight is an

⁷ The plot here is gain per 100 gm body weight (per cent gain) against lysine consumed per 100 gm body weight. Comparison of total gain against total lysine consumed presents the same general picture.

adequate measure of tissue protein content. Barnes et al. ('45) found that the maximum range of protein content of rat carcasses within a large range of protein levels and between proteins varying widely in value was "well within the experimental error of most biological assay methods with small numbers of animals." Results with chicks (Grau, '46) indicated that the phenylalanine level of the diet had no marked effect on the protein content of the carcass, and although poor diets produced carcasses with a low fat content, the effects

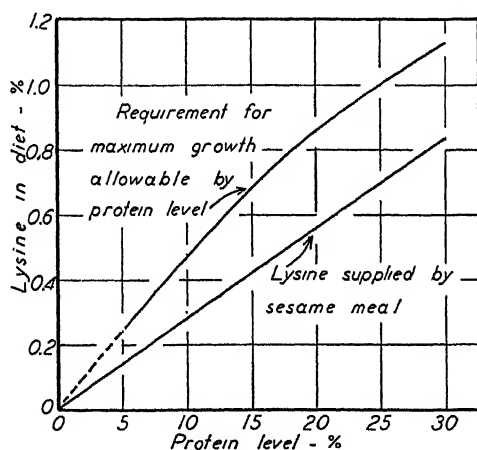


Fig. 5 The upper curve shows the relation of protein level to lysine requirement for maximum growth. The lower curve shows the level of lysine supplied by various levels of sesame meal protein.

on gain or loss of fat per day were negligible compared with gain or loss in weight.

In figure 5, the minimum lysine requirement for maximum growth which a particular level of protein will allow is plotted against the protein level, using the data from figure 3. Also shown in the same figure is the lysine supplied by various dietary levels of sesame meal protein. It should be noted that while the lysine requirement increases as the protein level is raised from 20% to 30%, this change causes no effect on the maximum growth rate (fig. 2).

The question of the availability of lysine in sesame meal must be considered as a possible cause for the results obtained. Except for the unlikely possibility that the proportion of available lysine varies with the protein intake, any effect of low digestibility of the protein or unavailability of the lysine would affect all protein levels by shifting the curves of figure 3 to the left. Some requirement difference for maximum growth would remain, however, because the curves have different slopes. Furthermore, the lysine requirement at the 20% protein level found here agrees very well with data obtained with other protein sources (Almquist, '47). Perhaps the strongest evidence in favor of the high availability of lysine in sesame meal rests on the slope of the curves at lysine levels below the minimum for best growth. Complete lack of an essential amino acid causes a loss in weight of about 3% per day under conditions such as these, and from this point the curve of growth rate against amino acid level rises along a straight line to optimum growth (Almquist, '47). If an appreciable portion of the lysine present in sesame meal were unavailable, the slope of these curves would have to be greater in order to approach a loss of 3% per day at 0 lysine. Extrapolation of the curves of figure 3 shows that they intersect the ordinate at growth rates of between -1.5 and -3% per day. Thus the level of lysine available to the chick must be close to the total calculated lysine level.

The results presented above indicate clearly that the lysine requirement must be defined in terms of the protein level of the diet. These results cannot yet be generalized to include all the other essential amino acids; however, the chick requirement for methionine has been found to increase as the protein level is increased.

A protein such as that of the sesame seed, which does not satisfy the lysine requirement when fed at the 20% protein level, may be fed at a higher level in order to approach the satisfaction of the requirements, but at the higher protein level the lysine requirement is higher; hence, the lysine will be less efficiently used, as will all the other amino acids.

The application of these results to the biological assay of lysine in proteins indicates that the addition of proteinaceous material containing an unknown amount of lysine to a diet deficient in lysine will give an estimate of the lysine content below the actual value. For example, if sesame meal is to be assayed, and the 20% protein curve of figure 3 serves as the basis of the assay, the growth obtained by adding 10% sesame meal protein will be the same as that obtained with the unsupplemented 30% protein level. When the observed growth rate (4.8%) is projected on the 20% curve and the lysine content of the diet is read off, the apparent lysine level of the diet will be found to be 0.67%. Since 20% sesame protein contains 0.56% lysine, 0.11% was apparently added by the 10% sesame protein. But 10% of sesame protein actually contains 0.28% lysine; thus the bioassay is in error by 0.17% of the diet, which is 61% of the actual amount. If the bioassay were correct, either the requirement for or the availability of the lysine, or both, would have to be much smaller. The arguments against these possibilities have been pointed out above.⁸

It has been reported recently that high casein levels increase the need for supplementary arginine in chick diets (Hill and Van Poucke, '47), but whether this effect is of the same type as that reported here cannot be stated until further experiments have been conducted.

SUMMARY

Various levels of sesame meal protein and lysine have been fed to young chicks, and the effects on growth and feed consumption have been observed. It was found that as the protein level was increased, the lysine requirement for maximum growth at a particular protein level increased. This was true whether the lysine requirement was expressed as percentage of the diet, or as the weight of lysine consumed per day per unit of body weight.

* Methionine bioassays showed no such discrepancy between chemical and biological methods of analysis when the protein level was kept constant (Grau and Almquist, '45).

Amino acid requirements may best be expressed in terms of percentages of the diet at a particular protein level, preferably the minimum protein level necessary to promote rapid growth.

While higher levels of sesame protein provide higher levels of lysine, this additional lysine is inefficiently used, because the requirement for it is also increased. Other amino acids are also inefficiently used, because the protein level is above the optimum.

A lysine bioassay procedure in which the amino acid content of proteinaceous material is determined by addition to a sesame meal diet will produce erroneous results unless corrections are made for the increased lysine requirement with increased protein levels.

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THE DETERMINATION OF THE MINIMUM NITROGEN REQUIREMENT OF THE ADULT DOG FOR MAINTENANCE OF NITROGEN BALANCE

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NINETEEN FIGURES

(Received for publication February 20, 1948)

Rose and Rice ('39) have shown that, in order to maintain positive nitrogen balance in adult dogs, a certain minimum quantity of each of the 9 essential amino acids must be administered daily. Thus, in part at least, a single indispensable amino acid, if present in smaller amount than the rest in relation to the quantitative requirements of the animal, may determine the utilization of a mixture. A well-known illustration of this fact is the increased growth of young rats obtainable by adding either cystine or methionine to diets in which less than an optimum amount of casein is the sole source of protein (Osborne and Mendel, '13; Cox, Mueller, Elman, Albanese, Kenmerer, Barton and Holt, '47). In this case, methionine is the amino acid limiting the utilization of this protein. Thus, the mixture supplying the amino acids in the respective proportions most nearly meeting the animal's quantitative requirements should maintain positive balance at the lowest level of nitrogen intake.

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With this in mind, experiments have been carried out to determine the minimum quantity of nitrogen which must be administered in the form of various proteins and hydrolysates to maintain positive nitrogen balance. The effect of adding methionine to casein and to an acid hydrolysate of casein has also been studied. Data so obtained make possible quantitative comparison of the efficiency of various amino acid sources for the maintenance of positive nitrogen balance. The protein, or mixture of amino acids, which maintains positive nitrogen balance at the lowest level of nitrogen administration probably contains the amino acids in proportions most nearly approximating the requirements of the animal. When the quantitative requirements of the species are known, the amount of nitrogen, in the form of various proteins or amino acid mixtures, necessary for maintenance will be an excellent measure of the biological value of the material.

The procedure and diet employed were the same as reported previously (Kade et al., '46). At all times approximately 75 cal. per kilogram of body weight were fed as a practically nitrogen-free diet, which contained approximately 60 mg of nitrogen per 100 gm of food, of which 40 mg originated in the vitamins added. Thus, the diet contributed approximately 10 mg of nitrogen per kilogram of body weight which was always taken into account in determining the total nitrogen fed. The amino acids were administered by incorporation in the food or separately by stomach tube. All test periods were 7 days, but the urine was analyzed daily. This was done primarily to follow the trend of nitrogen excretion. In order to eliminate the necessity of altering the quantity of nitrogen being fed as the weight of the animal varied, an optimum body weight at a nutritive state of 0.30 was estimated for each test dog by the Cowgill and Drabkin ('27) formula $W = (0.3L)^3$, where W = weight in kilograms and L = length in centimeters.

The following experiments gave the data presented in the accompanying graphs. Each graph represents a separate determination of the minimum quantity of nitrogen in the form

of a protein or hydrolysate necessary to maintain equilibrium. All the animals were in the same nutritive condition. Consistent and reproducible values can be obtained only when the dogs are carefully brought to this standard condition of minimum nitrogen metabolism. This is accomplished, as outlined in the previous paper, by feeding a 7% casein diet at a nitrogen intake of 150 mg per kilogram for several weeks.

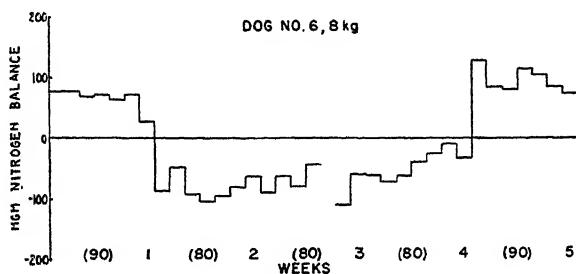


Figure 1

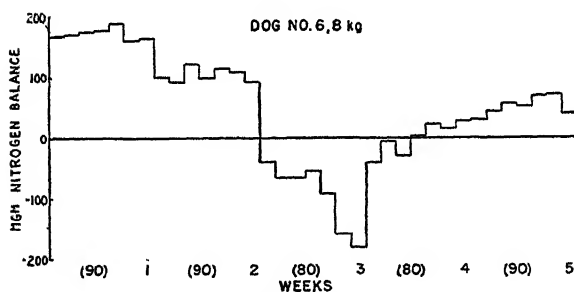


Figure 2

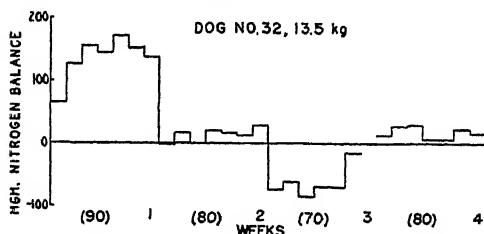


Figure 3

Figs. 1, 2 and 3 Nitrogen balance with lactalbumin as the sole source of amino acids. The figures in parentheses indicate the daily nitrogen intake per kilogram of body weight during that week.

DETERMINATION OF THE MINIMUM NITROGEN INTAKE
NECESSARY FOR MAINTENANCE OF POSITIVE
BALANCE IN THE ADULT DOG

*Experiment 1. Lactalbumin.*² The data presented in figures 1, 2 and 3 show that approximately 80 to 90 mg of nitrogen per kilogram of body weight, furnished as this lactalbumin, is sufficient for maintaining nitrogen balance in the adult dog.

*Experiment 2. Blood fibrin.*³ The data of figures 4 and 5 show that approximately 100 to 110 mg of nitrogen per kilo-

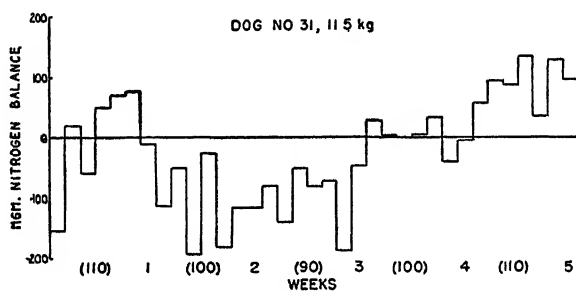


Figure 4

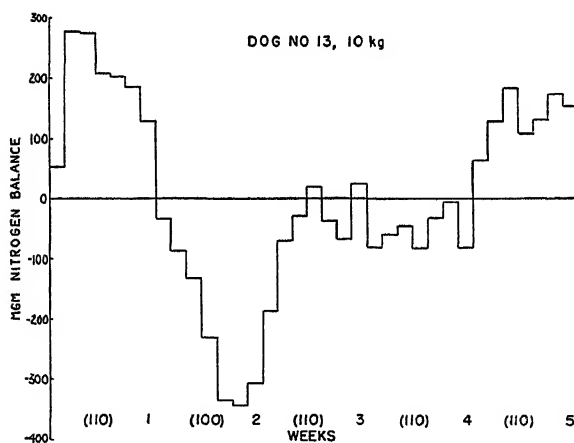


Figure 5

Figs. 4 and 5 Nitrogen balance with blood fibrin as the sole source of amino acids. The figures in parentheses indicate the daily nitrogen intake per kilogram of body weight during that week.

² Borden's lactalbumin 15-42, 12.6% nitrogen.

³ Wilson Laboratories blood fibrin, 15.5% nitrogen.

gram of body weight, furnished as this blood fibrin, is sufficient for maintaining nitrogen balance in the adult dog.

*Experiment 3. Casein.*⁴ The data in figures 6 and 7 show that approximately 130 to 140 mg of nitrogen per kilogram of body weight, furnished as this casein, is sufficient for maintaining nitrogen balance in the adult dog.

*Experiment 4. Casein supplemented with adequate amounts of methionine.*⁵ The data in figures 8 and 9 show that approxi-

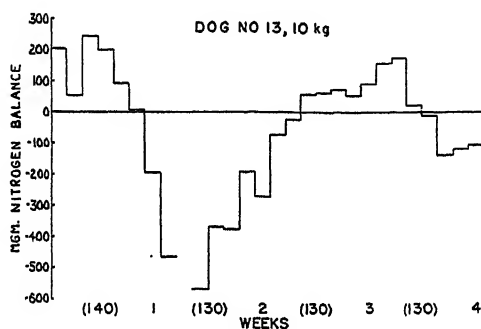


Figure 6

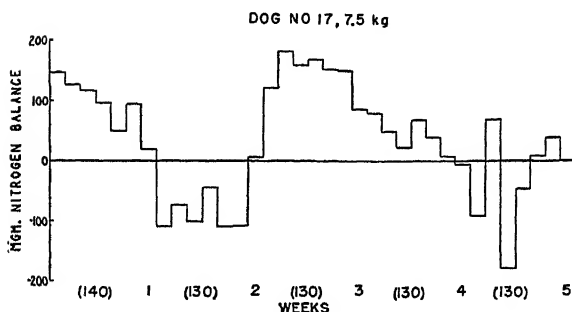


Figure 7

Figs. 6 and 7 Nitrogen balance with casein as the sole source of amino acids. The figures in parentheses indicate the daily nitrogen intake per kilogram of body weight during that week. During the third week 20 mg of additional methionine was added per day.

⁴ Sheffield new process casein, 14.14% nitrogen.

⁵ Sufficient methionine added to bring total daily methionine intake to 53 mg per kilogram of body weight. Casein was assumed to have 3.2% methionine (Block and Bolling, '45).

mately 90 mg of nitrogen per kilogram of body weight, furnished as casein supplemented with adequate amounts of methionine, is sufficient for maintaining nitrogen balance in the adult dog.

Experiment 5. Hydrolysates of casein. The data of figures 10, 11, 12, 13 and 14 show that approximately 130 to 140 mg of

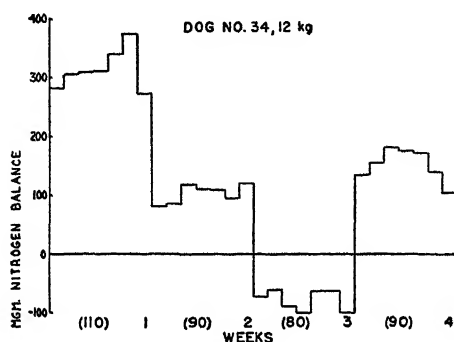


Figure 8

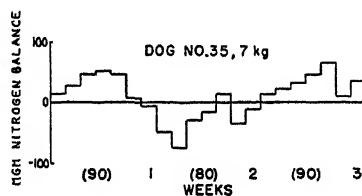


Figure 9

Figs. 8 and 9 Nitrogen balance with casein supplemented with sufficient methionine to bring the total daily methionine intake to 53 mg per kilogram of body weight. Casein was assumed to have 3.2% methionine (Block and Bolling, '45). The figures in parentheses indicate the daily nitrogen intake per kilogram of body weight during that week.

nitrogen per kilogram of body weight, furnished as hydrolysates of casein prepared by enzymatic hydrolysis, partial acid hydrolysis or complete acid hydrolysis and fortified with tryptophane, is sufficient for maintaining nitrogen balance in the adult dog.

Experiment 6. An acid hydrolysate of casein⁶ supplemented with adequate amounts of tryptophane and methionine. The data in figures 15, 16, 17 and 18 show that approximately 90 mg of nitrogen per kilogram of body weight, furnished as a partial acid hydrolysate of casein and supplemented with adequate

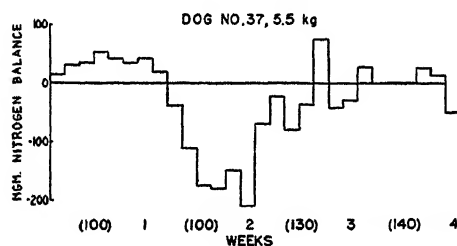


Figure 10

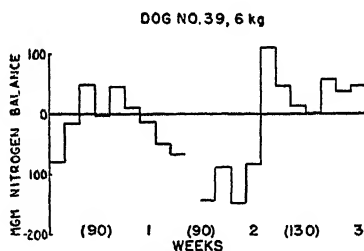


Figure 11

Figs. 10 and 11 Nitrogen balance with a partial acid hydrolysate of casein (Sahyun, Kade and Houston, '47) supplemented daily during the first week with 20 mg per kilogram of body weight of DL-tryptophane and 35 mg per kilogram of body weight of DL-methionine, and supplemented daily during the second, third and fourth weeks with 20 mg per kilogram of body weight of DL-tryptophane. The figures in parentheses indicate the daily nitrogen intake per kilogram of body weight during that week.

quantities of methionine and tryptophane, will maintain nitrogen balance in the adult dog.

Figure 19 summarizes briefly the minimum nitrogen requirements of the adult dog for the maintenance of nitrogen balance when the nitrogen is furnished by the tested proteins or hydrolysates.

⁶Sheffield new process casein, 14.14% nitrogen.

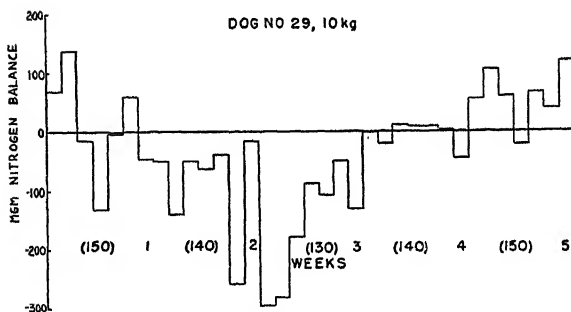
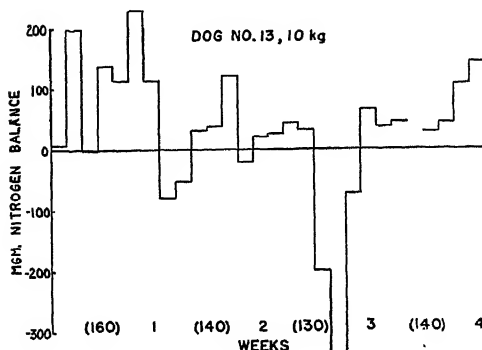
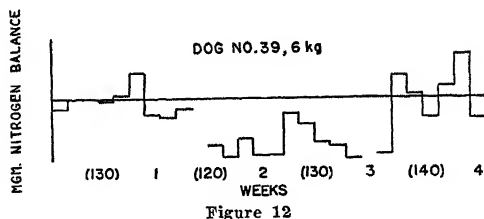


Fig. 12 Nitrogen balance with a partial acid hydrolysate of casein (Sahyun, Kade and Houston, '47) supplemented daily with 20 mg per kilogram of body weight of DL-tryptophane. The figures in parentheses indicate the daily nitrogen intake per kilogram of body weight during that week.

Fig. 13 Nitrogen balance with a complete acid hydrolysate of casein (Sahyun, '41) supplemented daily with 20 mg per kilogram of body weight of DL-tryptophane. The figures in parentheses indicate the daily nitrogen intake per kilogram of body weight during that week.

Fig. 14 Nitrogen balance with an enzymatic digest of casein (B-C-4-13) as the sole source of amino acids. The figures in parentheses indicate the daily nitrogen intake per kilogram of body weight during that week.

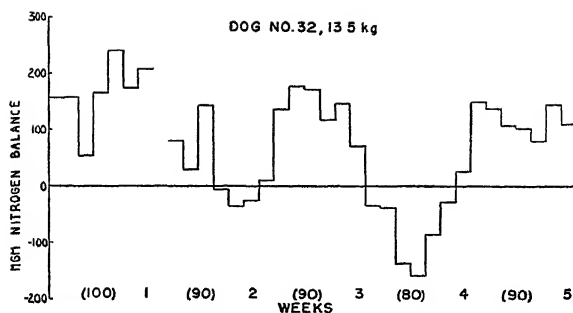


Figure 15

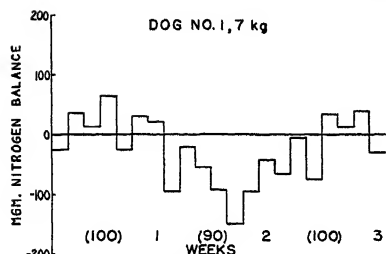


Figure 16

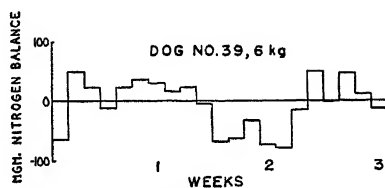


Figure 17

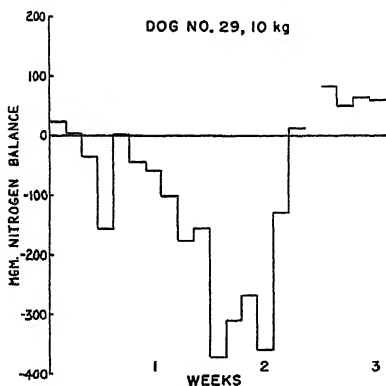


Figure 18

Figs. 15 and 16 Nitrogen balance with a partial acid hydrolysate of casein (Sahyun, Kade and Houston, '47) supplemented daily with 20 mg per kilogram of body weight of DL-tryptophane and sufficient DL-methionine to bring the total daily methionine intake to 53 mg per kilogram of body weight. The hydrolysate was assumed to have 3.2% methionine. The figures in parentheses indicate the daily nitrogen intake per kilogram of body weight during that week.

Figs. 17 and 18 Nitrogen balance with a partial acid hydrolysate of casein fed at a level of 90 mg per kilogram of body weight per day and supplemented daily during the first and third weeks with 20 mg per kilogram of body weight of DL-tryptophane and sufficient DL-methionine to bring the total daily methionine intake to 53 mg per kilogram of body weight. The hydrolysate was assumed to have 3.2% methionine. During the second week the methionine was omitted.

Considerably more nitrogen is required to maintain equilibrium when it is fed in the form of casein than in the form of lactalbumin. However, if the casein is supplemented with methionine, the requirement is approximately the same as for lactalbumin.

Casein hydrolysates prepared by enzymatic or acid degradation, either completely or only partially hydrolyzed, are equal in their ability to maintain nitrogen equilibrium in the adult

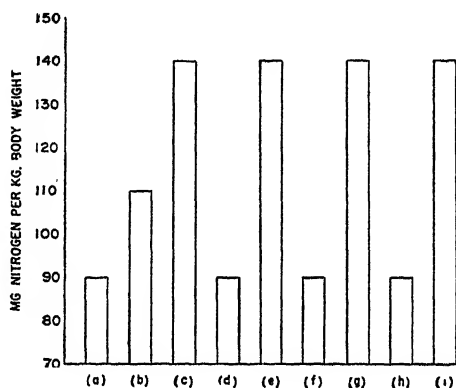


Fig. 19 Minimum quantity of nitrogen, furnished as protein or hydrolysates of protein, necessary for maintenance of nitrogen balance in the adult dog: (a) lactalbumin, (b) fibrin, (c) casein, (d) casein plus methionine, (e) complete acid hydrolysate of casein plus tryptophane, (f) complete acid hydrolysate of casein plus tryptophane and methionine, (g) partial acid hydrolysate of casein plus tryptophane, (h) partial acid hydrolysate of casein plus tryptophane and methionine, and (i) enzymatic hydrolysate of casein.

dog if the tryptophane destroyed in processing is restored. These hydrolysates are equivalent to the native casein in the maintenance of nitrogen balance. Approximately 140 mg of nitrogen per kilogram of body weight must be furnished, either as the native casein or as hydrolysates prepared from it. Adding adequate methionine to any of these hydrolysates of casein or to the casein itself lowers the nitrogen requirement to 90 mg per kilogram of body weight.

DISCUSSION

While this study was in process, a publication by Risser ('46) appeared in which much the same method was described. In general, the results reported here are in agreement with those of Risser. The discrepancies in the approximate values obtained for casein and fibrin can probably be ascribed to the differences in methods used for determining the standard weight of the animal and for calculating the amount of nitrogen fed. Risser used the kennel weight of the dog, but the experience in our laboratory is that the animals are always underweight when received and that after a few weeks on this diet they gain significantly.

In spite of adjusting the food intake to maintain a steady nutritive state, all animals on these diets continued to gain weight though more slowly in negative nitrogen balance. The rate of increase became very small after the dog reached a nutritive state of 0.30. No changes in weight over weekly periods as large as those reported by Risser were observed.

Since the animals obtained approximately 10 mg of nitrogen per kilogram of body weight from the basic diet, primarily as the vitamins, the actual nitrogen intake from the amino acid source was less by this amount than the total nitrogen intake indicated. However, since the balance is based on the difference between intake and excretion, and the non-amino acid nitrogen is constant and necessary, all results are expressed on the basis of total nitrogen required to maintain balance. If this 10 mg of nitrogen per kilogram of body weight were subtracted from values reported here, there would be no significant differences between these results for casein and fibrin and those reported by Risser.

Risser ('46) suggests that the difference in the utilization of casein and fibrin might be due to "incomplete hydrolysis of the protein in the digestive tract." In this connection it is interesting to note that a complete hydrolysate, the partial acid hydrolysate, or an enzymatic hydrolysate of casein is no better than the casein itself. This tends to eliminate the possibility that the sulfur-containing amino acids in casein are

not liberated at a sufficiently rapid rate to be fully utilized. It would be a most unusual coincidence if the destruction of the sulfur-containing amino acids and removal in the preparation of these different hydrolysates should be exactly equal to "the slower enzymatic release." Moreover, the fact that the addition of the methionine to casein and to its hydrolysate produces almost exactly the same effect seems to indicate that it is the pattern of amino acids rather than incomplete hydrolysis which accounts for the lower utilization of casein in comparison with fibrin or lactalbumin.

It is unlikely that streptogenin or other unidentified weight growth factors (Sprince and Woolley, '45; Rose and Womack, '46) play any role in the utilization of these proteins and hydrolysates in the adult animals since there probably are none present in the complete acid hydrolysate which by this test is equivalent to the native casein which does contain streptogenin.

It is important to note that all these minima are for adult animals. It is entirely possible that the minimum levels of these proteins or hydrolysates will not furnish an adequate amount of some one of the amino acids required by the growing animal. In addition, the growing animal's need for one or more amino acids in particular may be so much larger that the limiting amino acid in the utilization of the mixture may not be the same. Thus, the relative values of proteins may not be the same for growing as for adult animals.

Species differences in amino acid requirements are also very likely to affect both the quantitative requirement and the relative value of the protein or mixture. However, data obtained on 1 species under 1 set of conditions will necessarily have qualitative meaning for other species whose amino acid requirements are qualitatively similar.

SUMMARY

Since the biological value of any protein or mixture of amino acids will be dependent, at least in part, on the amino acid that is present in the lowest proportion to its quantitative re-

quirement, it is possible to determine the relative values of nitrogen sources by determining the minimum quantity necessary for maintenance of nitrogen balance. It has been shown that only 90 mg of nitrogen per kilogram of body weight is required in the form of lactalbumin, and 140 mg of nitrogen per kilogram of body weight as casein, a partial acid hydrolysate of casein fortified with tryptophane, a complete acid hydrolysate of casein fortified with tryptophane, or an enzymatic hydrolysate of casein. However, when fortified with adequate amounts of methionine, either casein itself or the hydrolysates will maintain nitrogen balance with only 90 mg of nitrogen per kilogram of body weight.

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THE ENERGY EXPENDITURE OF 9- TO 11-YEAR- OLD BOYS AND GIRLS (1) STANDING DRAWING AND (2) DRESSING AND UNDESSING

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The need for more accurate information concerning the energy expenditure of boys and girls of all ages is felt whenever the problem of recommending Calorie allowances for children arises. Most of our present knowledge of the energy needs of boys and girls has come from studies of food consumption in relation to gains in height and weight. As noted by Taylor, Lamb, Robertson and MacLeod ('48), few measurements of the actual energy expenditure of children have been made as compared with the large number on adults. That children spend more energy per kilogram of body weight in certain activities than do adults engaged in the same activities has been found in studies conducted in this laboratory. Thompson ('40), comparing the energy expenditure of young girls during quiet play and cycling with that of college women engaged in the same activities under identical conditions, found the energy cost of quiet play (exclusive of the basal metabolism) for the girls to be more than 3 times that for the women and the difference for cycling was of about the same order of magnitude. Similar differences for boys and

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college men were found by Lamb ('42). Such comparisons emphasize the need for more accurate information concerning the energy requirements of children.

Since differences in the daily activities of children begin to be more varied at an age when physical development and muscle coördination are such that they can and do perform many of the same tasks as adults, the age range of 9 to 11 years was selected as the starting point for an extensive study of the energy expenditure of boys and girls in a number of activities. A survey was made of the activities engaged in by a large number of 9- to 11-year-old boys and girls living under different conditions.² As was anticipated, this survey revealed a great variety of activities which need to be investigated. It was decided to investigate first those which are common to most children. This paper reports the energy expenditure of 9- to 11-year-old boys and girls when standing drawing (an activity common to all school children who work at blackboards) and when dressing and undressing.

SUBJECTS

Seven boys and 12 girls, ranging in age from 9 to 11 years, were selected as experimental subjects. Some of these children attended a private school and lived at home with their parents, and some came from a children's home where they lived under more routine conditions. Health records reported by physicians were studied to ascertain the health status of the children and to make sure that they had no physical defects and might be considered average normal individuals. The weight, height, surface area and deviations from predicted weight for height and age (Baldwin-Wood standards) were determined. The average values are given in table 1.

PROCEDURE

The activity studies were carried out in the open-circuit respiration chamber described by Taylor, Lamb, Robertson

² Findings of this survey will be reported at another time.

and MacLeod ('48). The total weight of carbon dioxide produced during an experimental period was determined by analysis of a sample of the chamber air at the beginning and at the end of the period and adding the difference to the increase in weight of the absorbing train during the period. The Carpenter modification of the Haldane gas analysis apparatus ('23, '29) was used for determining the carbon dioxide content of the chamber air. The carbon dioxide produced by 2 children during an experimental period was divided by 2 to obtain the average carbon dioxide production per child.

Two walls of the chamber were covered with large sheets of drawing paper placed at blackboard height, and pictures, which could be copied or colored, were also placed on the walls to provide inspiration for those children whose imaginations needed a little stimulation. One wall of the respiration chamber was fitted with 2 small mirrors placed at suitable height so that the children could comb their hair without any undue stooping or stretching. The activity studies were conducted in the afternoon after school and the children were asked to bring gymnasium clothes for the dressing and undressing activity.

On arrival in the laboratory, the children placed their clothes in small suitcases and each child was given a box of colored crayons and a game or puzzles to take into the chamber. Two boys or 2 girls were studied at a time. In this way it was possible to keep the children in the respiration chamber for a shorter period of time and still collect sufficient carbon dioxide to maintain a satisfactory degree of accuracy. Also, for an experimental period lasting 35 minutes, it was more fun for the children when 2 of them could be subjects at the same time. Before entering the chamber they were given detailed instructions regarding the procedure for the test.

After entering the chamber they sat quietly for a 10-minute rest period during which time they were permitted to play Chinese checkers or to work on "Goofy" puzzles. They were then given the signal to start drawing and this activity was continued for a period of 15 minutes. They were free to draw

TABLE 1

*Average weight, height, deviation from predicted weight, surface area and basal metabolism of subjects
(figures in parentheses show range)*

	WEIGHT		HEIGHT		DEVIATION FROM PRE- DICTED WEIGHT	SURFACE AREA	BASAL METABOLISM IN CALORIES PER			
	lb.	kg	in.	cm			%	24 hr.	kg/hr.	m ² /hr.
<i>Boys</i>										
7 subjects	77.2	35.1	55.1	139.9	+ 5.0	1.16	1192	1.42	42.8	8.5
Age range	(69.3-	(31.5-	(52.3-	(132.8-	(- 5.4-	(1.06-	(1049-	(1.28-	(39.3-	(7.7-
9-8 to 10-8 ¹	84.5)	38.4)	57.9)	147.0)	+ 16.2)	1.25)	1391)	1.57)	46.7)	9.6)
<i>Girls</i>										
12 subjects	73.9	33.6	55.1	139.9	- 1.1	1.15	1186	1.49	43.0	8.4
Age range	(59.6-	(27.1-	(51.0-	(129.6-	(- 10.3-	(0.99-	(914-	(1.29-	(37.7-	(6.7-
8-10 to 11-5 ¹	96.6)	43.9)	59.1)	150.1)	+ 11.0)	1.35)	1473)	1.69)	48.1)	9.8)

¹ First number in each set refers to years, the second to months.

anything they wished but were asked to keep actively engaged in standing drawing throughout the 15-minute experimental period. At the end of the drawing period they were given the signal to start undressing and dressing which was continued for 10 minutes. During this period they changed their clothes, getting into gymnasium clothes and back again into their regular school clothes, and changed their shoes and combed their hair. Most of the children carried out this routine more than once during the 10-minute period allotted to dressing and undressing.

Basal metabolism determinations were made on all subjects by means of the Benedict-Roth and the Sanborn-Benedict respiration apparatus, under generally accepted standard conditions. Two determinations were made on each child for as many mornings as were required to obtain the average of at least the 3 lowest figures checking within 5%. Usually 2 mornings sufficed. Basal metabolism determinations and activity studies were made on each child within a 6-month period. A summary of the basal metabolism determinations is given in table 1. In each case the basal metabolism of the children included in the study agreed within the commonly accepted normal range of $\pm 15\%$ with 2 or more of the standards currently used for the basal metabolism of children (Dreyer, '20; Boothby, Berkson and Dunn, '36; Talbot, '38; Lewis, Duval and Iliff, '43).

RESULTS AND DISCUSSION

The average results of the activity studies are summarized in table 2. Eleven cases were obtained on boys and 14 cases on girls for each of the activities, and the results expressed in terms of total Calories per hour per kilogram of body weight and per centimeter of height.

Standing drawing

The average energy expenditure for standing drawing by the boys was found to be 3.19 ± 0.10 Cal. per kilogram of body weight per hour and 0.83 ± 0.03 Cal. per centimeter of

height per hour, and by the girls, 2.62 ± 0.05 Cal. per kilogram of body weight per hour and 0.63 ± 0.01 Cal. per centimeter of height per hour. That the boys spent more energy than the girls in performing the same activity is possibly accounted for by the fact that in general the boys in drawing tended to

TABLE 2

Average energy expenditure of children (1) standing drawing and (2) dressing and undressing (including the basal metabolism)

	STANDING DRAWING		DRESSING AND UNDRRESSING	
	Boys	Girls	Boys	Girls
<i>CO₂ produced/ child/hr. (gm)</i>				
Mean \pm P.E. ¹	38.89 \pm 1.69	29.19 \pm .64	50.35 \pm 1.97	46.47 \pm 1.35
No. of cases	11	14	11	14
C.V. ² (%)	21.7	12.1	19.3	16.1
P.E. as per cent of mean	4.4	2.2	3.9	2.9
<i>Cal./kg/hr.</i>				
Mean \pm P.E.	3.19 \pm .10	2.62 \pm .05	4.29 \pm .16	4.04 \pm .13
No. of cases	11	14	11	14
C.V. (%)	15.8	9.6	18.9	18.1
P.E. as per cent of mean	3.1	1.9	3.7	3.2
<i>Cal./cm ht./hr.</i>				
Mean \pm P.E.	0.83 \pm .03	0.63 \pm .01	1.09 \pm .04	1.00 \pm .03
No. of cases	11	14	11	14
C.V. (%)	20.5	9.5	18.3	14.9
P.E. as per cent of mean	3.6	1.6	3.7	3.0

¹ Probable error.

² Coefficient of variation.

work with more flourish than the girls. Their sketches were carried out on a large scale and they usually covered all available space provided for drawing, their pictures showing considerable initiative. On the contrary, the girls tended to confine their drawing to a much smaller space and there was a

stronger tendency simply to color the outline drawings provided than to make original sketches of their own. When given the opportunity to draw under identical conditions this difference between the boys and girls was found whether they came from the home for children or the private school. The difference is seen in the higher coefficients of variation for the boys, 15.8 and 20.5, respectively, as compared with 9.6 and 9.5 for the girls.

Compared with the basal metabolism, there is an increase in energy expenditure per kilogram per hour for standing drawing of 125% for the boys and 76% for the girls. There was no significant difference in the energy expenditure for this activity between the subjects coming from the home for children and those coming from the private school. The energy expenditure for standing drawing may be taken as fairly typical of that for boys and girls doing problems or drawing on the blackboard, or making murals, an activity quite common in progressive schools throughout the country.

Dressing and undressing

The average energy expenditure for dressing and undressing by the boys was found to be 4.29 ± 0.16 Cal. per kilogram of weight per hour and 1.09 ± 0.04 Cal. per centimeter of height per hour, and by the girls, 4.04 ± 0.13 Cal. per kilogram of weight per hour and 1.00 ± 0.03 Cal. per centimeter of height per hour. These figures indicate less difference between boys and girls in the energy they expend for dressing and undressing than for standing drawing. The coefficients of variation are 18.9% per kilogram of weight and 18.3% per centimeter of height for the boys, and 18.1% per kilogram of weight and 14.9% per centimeter of height for the girls. That coefficients of variation are higher for both boys and girls when dressing and undressing than when standing drawing is to be expected since the motions involved in dressing and undressing are so much more variable. For example, sometimes both boys and girls spent more time in combing their hair and the boys more in fixing their neckties than they did

at other times. The increase in energy expenditure per kilogram per hour for dressing and undressing over the basal energy expenditure amounts to 202% and 171% for boys and girls, respectively.

SUMMARY

1. The energy expenditure of 7 boys and 12 girls, 9 to 11 years of age, selected from a home for children and from a private school, has been measured while they were standing drawing and while they were dressing and undressing in a respiration chamber.

2. The average energy expenditure by the boys while standing drawing was found to be 3.19 ± 0.10 Cal. per kilogram of weight per hour with a coefficient of variation of 15.8% and 0.83 ± 0.03 Cal. per centimeter of height per hour with a coefficient of variation of 20.5%. The average energy expenditure by the girls while standing drawing was found to be 2.62 ± 0.05 Cal. per kilogram of weight per hour with a coefficient of variation of 9.6% and 0.63 ± 0.01 Cal. per centimeter of height per hour with a coefficient of variation of 9.5%.

3. The average energy expenditure by the boys while dressing and undressing was found to be 4.29 ± 0.16 Cal. per kilogram of weight per hour with a coefficient of variation of 18.9% and 1.09 ± 0.04 Cal. per centimeter of height per hour with a coefficient of variation of 18.3%. The average energy expenditure by the girls while dressing and undressing was found to be 4.04 ± 0.13 Cal. per kilogram of weight per hour with a coefficient of variation of 18.1% and 1.00 ± 0.03 Cal. per centimeter of height per hour with a coefficient of variation of 14.9%.

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THE BIOLOGICAL VALUE OF A MEAT HYDRO- LYSATE IN THE INFANT

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The practice of administering protein digests to infants suffering from diseases which cause excessive nitrogen loss seemed to us to make it imperative that the biological value of preparations derived from various proteins be assessed in the normal infant prior to their therapeutic application. Accordingly, in a previous publication, we (Albanese and associates, '47) reported that the nutritional values of an enzymatic digest of casein¹ and lactalbumin² were equal to that of a diluted evaporated milk formula and superior to a synthetic diet in which a tryptophane and cystine supplemented acid digest of casein constituted the principal source of nitrogen. In this report the nutritive characteristics of a synthetic milk containing an enzymatic digest of beef muscle as the protein component are compared with those of the evaporated milk formula.

EXPERIMENTAL

Diets and procedures

The composition and caloric distribution of the 2 formulae employed in this study are shown in table 1. These were given in 5 feedings daily at the rate of 100 cal. and 0.56 gm of

¹ Amigen.

² Edamin.

nitrogen per kilogram of body weight and were supplemented daily with 50 mg of ascorbic acid and 15 drops of oleum percomorphum. Owing to uncertainties regarding the B complex vitamins required by the infant, brewers' yeast was used instead of a mixture of the synthetically available components of this vitamin complex. The amino acids derived from this source can be readily ascertained from the literature (Carter and Phillips, '44). The nitrogen content of each diet preparation was determined by the micro-Kjeldahl

TABLE 1
Composition and caloric distribution of diets

Evaporated milk formula (EM diet):

Evaporated milk, 50 ml (70 cal.); corn sirup 10 ml (30 cal.); water 40 ml.
Total volume 100 ml; total calories 100.

Meat protein digest formula (MH diet):

Meat digest (Wilson's, ¹ 0.56 × 6.25)	3.5 gm	14.0 cal.
Mead Johnson Lab. Prod. no. 217	18.0 gm	
Fats	4.0 gm	36.0 cal.
Carbohydrates	11.6 gm	46.5 cal.
Mead Johnson Brewers' Yeast	1.0 gm	3.5 cal.
Water	88.0 ml	
Total	100.0 ml	100.0 cal.

Salt supplement mg per 100 ml: FeSO₄, 10; NaCl, 100; Ca gluconate, 790;
KH₂PO₄, 323; Ca(OH)₂, 197; K₂HPO₄, 108; KCl, 90; MgO, 18.

¹ We are indebted to Dr. C. E. Graham, of the Wilson Laboratories, for a supply of this preparation.

method and the daily N intake of the subjects calculated from the consumption record. The diets were tested in 5 normal male infants (2.5-8 months of age) for 4 consecutive 7-day periods. Collection of the excreta was omitted on week-ends to avoid complications which might arise from constant use of restraints. Twenty-four hour urine specimens were collected by means of adapters in bottles containing 10 ml or 15% (by volume) HCl and 1 ml of 10% alcoholic thymol. The feces were collected in 19 cm porcelain evaporating dishes held in place by

a fitted excavation in the mattress and subsequently accumulated under refrigeration for each diet period in jars containing 200 ml of 70% alcohol. The infants were weighed and submitted to complete clinical examinations daily during the course of the study.

Data on the nitrogen retention were obtained by subtracting from the daily N intake the total N output as found by micro-Kjeldahl analyses of the daily 24-hour urine collections and period pools of the feces.

The effect of the dietary changes on the blood proteins was ascertained by analyses of blood samples (5 ml) collected on the last day of each diet period. The hemoglobin content of these specimens was determined colorimetrically by the acid hematin method in the Klett-Summerson photoelectric colorimeter. The total plasma proteins, albumin/globulin ratios and non-protein N were measured by the techniques previously described by us.

Results

The nitrogen retention and body weight gains induced by the evaporated milk formula and meat hydrolysate preparation indicate that by these criteria the 2 diets are of equal nutritional quality (table 2). Moreover, the observed nitrogen retention values fall well within the range of those previously reported by ourselves and other workers using a variety of milk formulae (Albanese and associates, '47).

Attention is called to the observation that a shift in the N intake of subject A.H. from 0.7 to 0.6 gm per kilo on either diet had no appreciable effect on the nitrogen retention and weight gain. This suggests that effects of hyperalimentation reported by Nelson ('30) become apparent only at lower nitrogen intake levels than were imposed in this instance.

The data on the total plasma protein levels given in table 2 furnish further evidence of the comparable biological value of the diets. No significant changes in the albumin-globulin ratios and hemoglobin concentration of the blood were observed.

The amounts of the essential and some non-essential amino acids provided per kilo of body weight by the meat hydrolysate diet were computed from our analyses of the meat digest and the available amino acid data on the brewers' yeast (Carter and Phillips, '44). These results and corresponding figures

TABLE 2
*Biological value of evaporated milk and meat hydrolysate formulae
in the normal infant*

SUBJECT	DIET	TOTAL N INPUT	DAILY BODY WEIGHT CHANGE	NITROGEN RETENTION	TOTAL PLASMA PROTEIN
		<i>gm</i>	<i>gm</i>	<i>mg/kg</i>	<i>gm %</i>
J.A.,	EM	2.58	+ 14	122	6.18
2.5 months,	MH	2.36	+ 21	139	6.06
4,218 gm	MH	2.36	+ 20	116	5.75
	EM	2.53	+ 21	177	6.15
R.M.,	EM	3.03	+ 21	118	5.29
4.0 months,	MH	2.73	+ 28	107	5.30
4,871 gm	MH	2.73	+ 25	102	5.12
	EM	2.84	+ 24	106	5.36
A.H.,	EM	5.22	+ 14	194	6.41
7 months,	MH	4.22	+ 26	187	6.60
7, 154 gm	MH	5.22	+ 21	205	6.50
	EM	4.66	+ 25	185	6.35
D.C.,	EM	3.86	+ 28	150	6.06
8 months,	MH	3.83	+ 14	155	6.55
5,309 gm	MH	3.86	+ 25	140	5.68
	EM	4.10	+ 18	140	6.04
W.G.,	EM	4.02	+ 14	161	5.82
8 months,	MH	4.21	+ 20	156	5.30
6,683 gm	MH	4.02	+ 21	157	5.62
	EM	3.97	+ 17	144	6.25

for the evaporated milk diet which were derived from Williamson's ('44) analyses of cow's milk are given in table 3. Examination of the values listed under the column heading (b)-(a) reveals that the meat hydrolysate diet furnishes more of all the essential amino acids except phenylalanine, isoleucine and

leucine. The low isoleucine content of the meat hydrolysate diet would not seem to constitute a limiting factor since the 102 mg of isoleucine provided by this diet is well above the 90 mg of isoleucine per kilo of body weight found by Albanese and coworkers ('48) to be required by the infant. Inasmuch as the 2 diets were found to be of equal biological value, it can be inferred that the phenylalanine and leucine deficits of the meat hydrolysate diet are negligible or are compensated

TABLE 3

*Amino acid intake of infants on the evaporated milk and meat hydrolysate diets
(all values are given in mg per kilo of body weight)*

AMINO ACID	EVAPORATED MILK	MEAT HYDROLYSATE FORMULA			
	(a)	Meat hydrolysate	Brewers' yeast	Total (b)	(b)-(a)
	mg	mg	mg	mg	mg
Arginine	127	185	21	206	+ 79
Cystine	27	40	7	47	+ 20
Histidine	63	115	19	134	+ 71
Isoleucine	167	86	16	102	- 65
Leucine	490	360	65	425	- 65
Lysine	200	288	32	320	+ 120
Methionine	99	104	13	117	+ 18
Phenylalanine	177	149	20	169	- 8
Threonine	151	209	25	234	+ 183
Tryptophane	47	87	6	93	+ 46
Tyrosine	172	91	21	112	- 60
Valine	171	205	22	227	+ 56

in part by the excess of the other essential amino acids. A comparison of the data secured in this study with those previously reported (Albanese and associates, '47) for the enzymatic digests of casein and lactalbumin suggests that the biological value of the hydrolysate employed in this investigation is equal to that of the milk protein preparations.

SUMMARY

It has been found that the nitrogen retention and weight gain of infants maintained on a synthetic milk, in which an enzymatic digest of beef muscle constituted the principal

source of nitrogen, were equal to those obtained on an evaporated milk formula fed at the same fluid, caloric distribution and nitrogen levels.

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THE RELATION BETWEEN STARVATION, METABOLIC ACIDOSIS AND CONVULSIVE SEIZURES IN RATS¹

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TWO FIGURES

(Received for publication February 4, 1948)

In the course of an investigation of the metabolic determinants of convulsive seizures in pyridoxine-deficient rats it was observed that pair-fed control rats receiving a qualitatively adequate but quantitatively inadequate diet had lower electroshock thresholds than comparable animals allowed to eat at will. Further investigation showed that the susceptibility of rats to electrically induced convulsions could be increased by restricting the caloric intake and rapidly decreased by refeeding. This result was not anticipated, for Geyelin ('21) claimed that fasting reduces the incidence of seizures in epileptic patients. The benefit of fasting has been attributed to the attendant metabolic acidosis, and Wilder ('21), Peterman ('24) and others have asserted that a ketogenic diet which produces metabolic acidosis reduces the incidence of seizures in epileptic children. (See Lennox and Cobb ['28] for a thorough review of the older clinical literature.) Because our preliminary laboratory observations did not seem to agree with clinical experience, the relation between starvation, metabolic acidosis and brain excitability in rats was reexamined with the results described below.

¹ This work was supported by the United States Public Health Service Research Grant 155-c for the Study of the Physiology and Therapy of Convulsive Disorders.

METHODS

Male rats of the Sprague-Dawley strain were used. Precautions were taken to insure an uninterrupted supply of water and relative constancy of room temperature, factors which in our experience have an important effect upon the electroshock threshold. Two stock diets were used. Diet A consisted of a commercial dog food² plus 15 gm of green vegetables per rat 3 times a week. Diet B consisted of a commercial calf meal³ with 3% brewers' yeast and 3% wheat germ added.

The electroshock thresholds were determined with a specially constructed constant-current apparatus utilizing Spiegel corneal electrodes. The duration of current was 0.2 seconds, and the intensity was increased by increments of 1.0 milliampere or less at intervals of 1 hour until a minimal clonic seizure occurred. Under our experimental conditions, the mean threshold of any group of normal rats was almost perfectly constant from day to day or over a period of several days.

In examining maximal seizure patterns, the same apparatus was used and the same duration of exposure, but the intensity of the current was 150 milliamperes.

Blood was obtained for acid-base studies by heart puncture in unanesthetized rats. Blood was drawn into a 2 ml syringe containing a drop of 1% heparin solution, and it was handled anaerobically by the method of Davenport ('47a). The plasma pH was measured with a glass electrode, and the total carbon dioxide content of the plasma was determined by the method of Van Slyke and Neill ('24). The bicarbonate concentration of the plasma in millimoles per liter was calculated using pK of 6.10 and carbon dioxide solubility coefficient of 0.0301.

Respiratory acidosis or alkalosis may be present at the time the blood samples are taken, because asphyxia may be induced by holding the rats for heart puncture, or hyper-

² Purina Dog Chow.

³ Known as "Calf Builder" and prepared by General Mills, Inc., Minneapolis, Minn.

ventilation may accompany their struggles. Nevertheless, the presence and extent of metabolic acidosis can be estimated by a valid graphical method. Blood samples from 10 normal rats having no respiratory acidosis or alkalosis were found to have an average pH of 7.41 and a plasma bicarbonate concentration of 24.3 mM/l. This was plotted as the normal point on a pH-bicarbonate diagram in which ordinates were plasma bicarbonate concentrations in mM/l and abscissae were pH units. The normal buffer curve of rat blood was found by determining the carbon dioxide absorption curve of rat blood, and when plotted on the pH-bicarbonate diagram it was found to be a straight line having a slope of -24.7 mM/l/pH unit. A line having such a slope was drawn through the normal point.

When the plasma bicarbonate and pH of any blood sample are plotted as a point on the diagram the vertical distance between the point and the buffer line is a measure of the extra fixed acid present in the blood in mM/l. The pH of the blood, if no respiratory acidosis or alkalosis is present can be estimated by drawing a line through the point parallel to the normal buffer line and reading the pH at which this line crosses the isobar representing a $p\text{CO}_2$ of 40 mm Hg. A complete description of the theoretical basis of this graphical method has been given by Davenport ('47b).

RESULTS

Starvation and electroshock threshold

Ten young rats having an average body weight of 124 gm were placed on diet A and allowed to eat at will. Their food consumption, which averaged about 15 gm per rat, was measured daily, and their body weights and electroshock thresholds were determined at intervals of several days. The mean body weights and electroshock thresholds over a period of 121 days were recorded as the solid lines in figure 1.

A second group of 10 young rats having an average body weight of 127 gm was placed on diet A, and in the first 85

days the rats were given 4 to 6 gm of food each per day, the amount given being chosen in order to keep their body weights constant. After the 85th day they were allowed to eat at will. Their mean body weights and electroshock thresholds

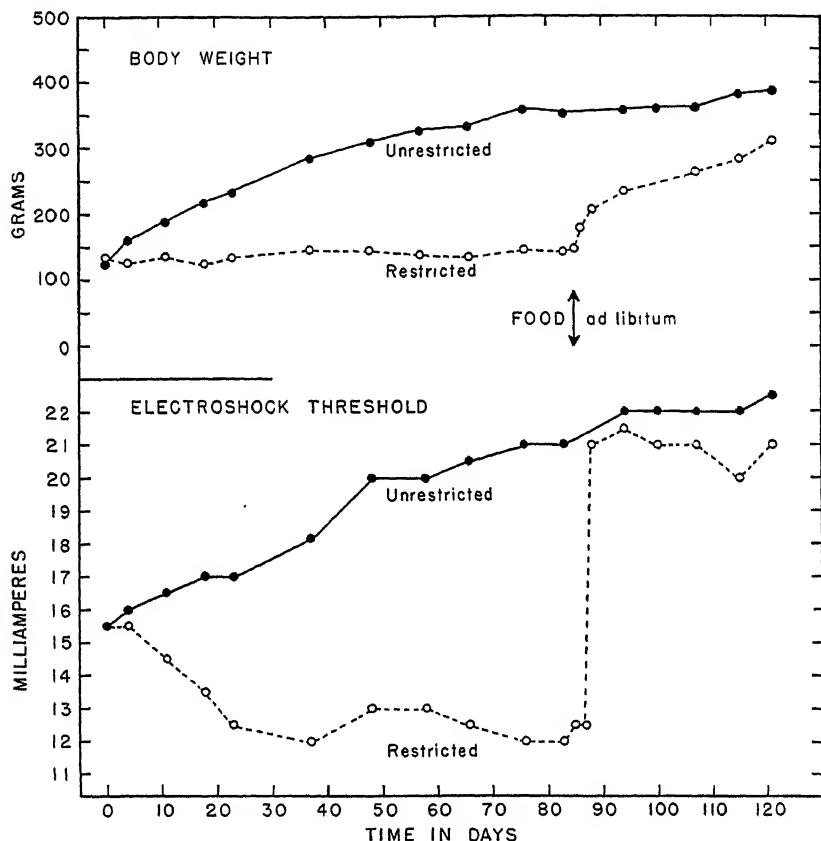


Fig. 1 Upper curves: average body weight of rats fed unrestricted (solid lines) and restricted (broken lines) amounts of diet A. Lower curves: average electroshock thresholds in milliamperes of the same rats.

over a period of 121 days are recorded as the broken lines in figure 1.

The electroshock thresholds of the rats on unrestricted food intake increased *pari passu* with the body weights. The body weights of the rats on restricted food intake remained

constant. Their electroshock thresholds fell during the first 23 days and thereafter remained significantly below the original thresholds. After the animals were allowed to eat at will their body weights began to increase slowly, but within 24 hours their electroshock thresholds rose abruptly to the level of the first group and remained high throughout the rest of the experiment.

The experiment was repeated twice with groups of rats of higher and lower initial weights, and the same results were obtained.

It is possible to raise or lower the electroshock threshold by varying the nutritional state of the rat as shown in figure 2. Ten rats whose average daily consumption of diet B was 15.5 gm each were given 5 or 6 gm per rat for 36 days. On the 37th and 38th days they were allowed to eat at will. Their food intake was again restricted until the 52nd and 53rd days when they were once more allowed to eat at will. During the initial period of food restriction the average electroshock threshold fell from 20.0 mA to 16.5 mA. Within 48 hours of the beginning of each period of unrestricted food intake the electroshock thresholds rose abruptly to the initial level and then fell slowly during the subsequent period of restriction.

Starvation and seizure pattern

The pattern of maximal seizures as well as the electroshock threshold is changed by starvation. Toman, Swinyard and Goodman ('46) have shown that the normal seizure pattern in rats is typical, consisting of a latent period, a period of tonic flexion, a period of tonic extension, a clonic phase which is frequently absent, and a period of post-seizure depression. The time from the application of the shock to the beginning of the extensor phase is 2.8 seconds and the time from the application of the shock to the end of the extensor phase is 14 seconds.

Rats having reduced electroshock thresholds as the result of starvation show the same sequence in maximal seizures as

do normal rats, but the time relationships are altered. The latent period is so short as to be almost undiscernible; the time to the beginning of the extensor phase is about 1.5 seconds; and the time to the end of the extensor phase is

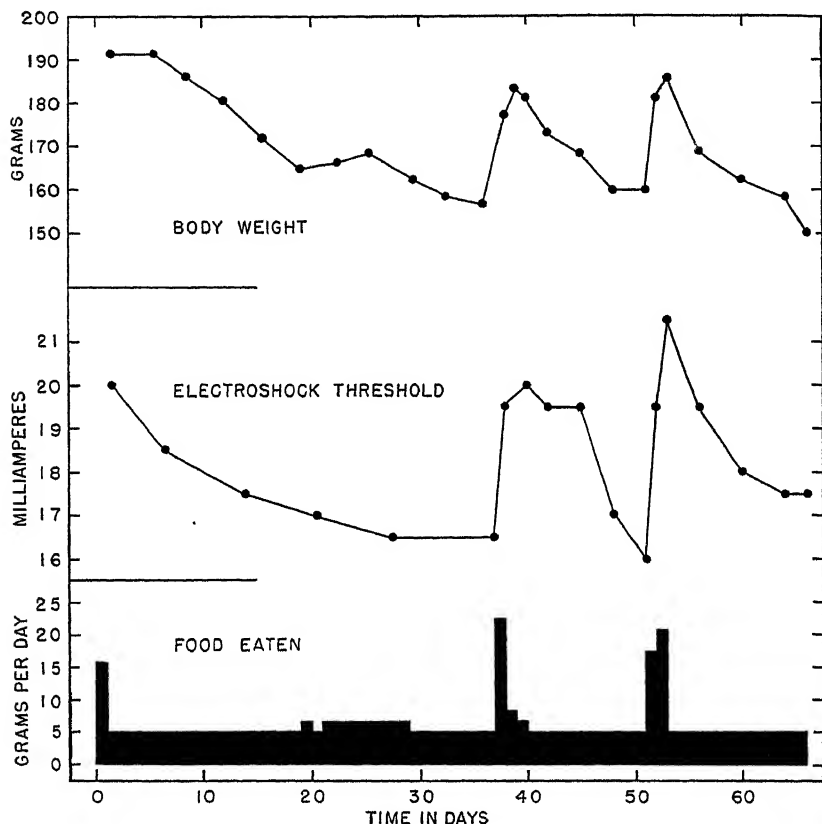


Fig. 2 Bottom bars: average amount of food eaten in grams per rat per day. Middle curve: average electroshock threshold in milliamperes. Upper curve: average body weight in grams.

lengthened to as much as 30 or 35 seconds. Unlike the electroshock threshold, the seizure pattern does not return entirely to normal within 48 hours after the animals are allowed access to unrestricted amount of food. A week or more of refeeding is required before the time relationships become normal.

Effect of dietary supplements

In order to determine which dietary component raises the electroshock threshold, control rats and rats on a restricted diet were allowed to eat the supplements listed in table 1. The control rats were given diet B in unrestricted amounts, and rats in another group were each given 5 gm daily of the same diet for 6 to 7 weeks before the supplements were fed. Both groups were provided with the supplements, with the exception of the vitamin mixture and the ammonium chloride, in unrestricted amounts for 2 days, and the amounts consumed were recorded. The electroshock thresholds were determined immediately before and 2 days after the beginning of the supplement.

The vitamin mixture was brought into aqueous solution by neutralization with sodium bicarbonate, and was given to the animals in 2 daily subcutaneous injections for 2 days. Each rat received the following amounts in milligrams daily: thiamine, pyridoxine, riboflavin and nicotinamide 1.0 each; calcium pantothenate 5.0; inositol 10.0; p-aminobenzoic acid 20.0; choline hydrochloride 20.0 and 2-methyl-naphthoquinone 0.1. The agar supplement was moistened purified bacteriological agar. The carbohydrate was commercial cornstarch, which

TABLE 1
Effect of dietary supplements on electroshock threshold.

SUPPLEMENT	UNRESTRICTED					RESTRICTED				
	No. of rats	Amount eaten	Electroshock threshold			No. of rats	Amount eaten	Electroshock threshold		
			Initial mA	Final mA	p ¹			Initial mA	Final mA	p ¹
		gm					gm			
Vitamins	5	..	21.8	22.0	0.4	10	..	17.1	17.2	0.6
Agar	20	5.2	23.9	23.8	0.7	20	9.2	17.2	17.3	0.9
Carbohydrate	10	35.8	23.3	23.5	0.5	10	31.0	17.1	20.5	0.01
Protein	10	15.8	23.0	23.0	0.9	19	19.0	16.8	19.7	0.01
Fat	20	15.8	22.9	21.0	0.01	21	19.2	17.9	19.2	0.01
Fat + 1% choline	10	16.2	24.2	22.4	0.01	10	17.7	16.7	18.9	0.01
NH ₄ Cl	12	0.5 mM/100 gm	19.0	19.0	0.9					
NH ₄ Cl	18	2.0 mM/100 gm	19.8	22.5	0.01					

¹ Fisher, '36.

The means by which these diverse substances raise the threshold is at present unknown. It is possible that a simple reduction of the calories available disturbs brain excitability and that restoration of a normal supply of calories allows a return to the normal state; but it is equally possible that the changes are caused by a much more devious mechanism. Further analysis of the phenomenon may reveal much about the relation between the brain's metabolism and its function.

The reduction of electroshock threshold in normal rats fed large amounts of fat cannot be explained without further investigation. These animals lost weight during the period of fat feeding, and their feces contained large quantities of fat. It is possible that the 2-day period of fat feeding actually reduced their caloric intake temporarily, but it is unlikely that so brief an interval of reduced intake would affect the electroshock thresholds.

Patton and his collaborators ('41) have shown that rats maintained on a diet which is qualitatively adequate but quantitatively inadequate have an increased incidence of audiogenic seizures. The fact that the susceptibility of rats to electrically induced seizures is similarly influenced by chronic undernutrition suggests that the 2 types of seizure may have a common basis. On the other hand, the same investigators (Patton et al., '42) found that the incidence of audiogenic seizures resulting from chronic underfeeding in rats could be reduced by the administration of a mixture of vitamins and minerals. This does not agree with our observation that massive doses of the vitamins of the B-complex have no effect on the electroshock threshold. One explanation for the discrepancy lies in the fact that our animals were treated with the supplement for only 2 days, whereas no effect was noticed on the incidence of audiogenic seizures until after 5 days except in rats which had previously received inadequate amounts of thiamine. More probably, our diet B contained such large portions of the vitamins of the B-complex that the animals were already receiving optimal amounts.

The extreme metabolic acidosis produced by the administration of 2.0 mM/100 gm ammonium chloride causes a significant rise in threshold, but it is unlikely that the less severe metabolic acidosis observed in the other instances is responsible for any change in excitability. Metabolic acidosis was present in the starved animals having lowered thresholds, but a similar degree of metabolic acidosis was also present in the starved animals when the threshold was raised by fat feeding, and in the normal animals when it was lowered by the same diet. Finally, ammonium chloride in a dose sufficient to produce a comparable metabolic acidosis is without effect on the threshold. It can be concluded that mild metabolic acidosis does not in itself change the threshold.

The results presented in this paper have 3 important practical bearings. The first is that the design and interpretation of experiments on convulsive phenomena must always take into account the nutritional state of the animals used. Any experimental procedure such as vitamin deprivation or extensive surgical intervention, if it reduces the food intake, may increase excitability. The second is that mild metabolic acidosis does not affect excitability; in experimental procedures in which a mild metabolic acidosis is incidentally produced, its effect can probably be disregarded. The third is that the evidence obtained on experimental animals does not agree with the clinical finding that starvation or a ketogenic diet reduces the incidence of seizures in epileptic patients. Not only is the electroshock threshold lowered by starvation, but the change in maximal seizure pattern is the direct opposite of that produced by clinically recognized antiepileptic drugs (Toman, Swinyard and Goodman, '46). In addition, our observations that the excitability may rise or fall in the face of similar degrees of ketosis show that ketosis itself is probably not responsible for the changes. In view of the unavoidable uncertainty of many clinical observations, the means by which starvation or ketogenic diets influence the course of epilepsy should be reexamined.

SUMMARY

1. The electroshock threshold of rats increases as their body weight increases.
2. If the caloric intake is restricted, the electroshock threshold falls significantly, and it rises abruptly to normal when the restriction is removed.
3. Supplemental administration of vitamins of the B complex or of agar to rats having low thresholds as a result of restricted caloric intake has no effect upon the electroshock thresholds.
4. Supplemental administration of carbohydrate, fat or protein to rats having low electroshock thresholds as the result of caloric restriction raises the threshold.
5. Supplemental administration of fat to normal rats lowers the electroshock threshold.
6. The electroshock threshold may either rise or fall when mild metabolic acidosis is produced by starvation or fat feeding.
7. Mild metabolic acidosis produced by ammonium chloride has no effect upon the electroshock threshold, but extreme metabolic acidosis raises the threshold.

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BIOLOGICAL VALUES OF SIX PARTIALLY-PURIFIED PROTEINS¹

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TWO FIGURES

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Because of conflicting data in the literature regarding the efficiency of utilization of proteins by different animal species, a coöperative endeavor by several different laboratories to resolve these discrepancies was proposed by the Bureau of Biological Research of Rutgers University. This laboratory was invited to assay the chosen proteins for their biological values by the maintenance method on human subjects.

The following proteins, all in the form of dry, granular or flour-like powders, were received directly from the committee, of which Dr. William H. Cole is chairman²: casein, egg albumen, whole egg, wheat gluten and peanut. The beef powder was received direct from Wilson and Company. All of the information available concerning the source and processing of the proteins was supplied by the committee.³

¹ This work was supported by a generous grant from the Williams and Waterman Fund of the Research Corporation of New York.

² The authors are greatly indebted to Dr. Cole and his committee for their patient and conscientious coöperation.

³ This information will be found in the addendum.

EXPERIMENTAL

The diet squad

It is much more difficult to select a homogeneous group of human subjects, than of rats or even dogs. There was only 1 period of the year, namely, from June 1st to September, when the laboratory facilities, necessary for a nutritional investigation of this sort, could be freed entirely from their use in whole or in part by medical students. The selection of subjects therefore had to be made just when medical students were leaving. At other times we might have been able to select a group composed entirely of men or of women. Fortunately there was a sufficient number of men and women graduate and medical students remaining through the summer as assistants in this and other departments so that it was possible to assemble a squad of 11 composed of 5 women and 6 men. One man was excused on the 16th day and this left an equal number of each sex for the 4 determinative periods beginning June 21st, 1947. Preceding this date the subjects had been subsisting for 15 days on the 3 to 5% (of calories) egg diet which has been used so much in this laboratory both as a reference standard (Sumner, Pierce and Murlin, '38; Murlin, Nasset and Marsh, '38; Murlin, Marshall and Kochakian, '41) and as a recovery and stabilizing diet (Murlin, Edwards, Hawley and Clark, '46). This period of time was usefully employed in training the subjects to comply rigidly with a code of rules which each person signed, indicating his understanding and acceptance of them. On the whole they lived up to the rules. It is human to forget, and small lapses were excusable, if they were not serious, because we always had a sufficient number of days of collection so that omitting now and then a day from the data could not materially affect averages.

In confirmation of the impressions of the senior author as to the soundness of their health, all the original participants were given a physical examination by Dr. Lyman C. Boynton, part-time instructor in medicine and formerly a member of the department staff. All of them qualified.

Six of the original 11 served as subjects for the entire series of 15 experimental periods comprising a total of 85 days. Besides the first person excused on the 16th day, 4 others were excused on the 42nd, 47th, 51st and 61st days, respectively, because of persistent nausea or emesis, or both, for some days previously. Some of them, 1 young woman in particular, persisted in taking the diets longer than was good for them. None of these 4 suffered any serious harm,⁴ however, and symptoms quickly disappeared when they returned to more normal diets. The very low protein diet which is called "no-protein" in this laboratory was the most difficult to endure; but the raw casein diet, because of its harsh, sandy roughness and utter lack of flavor, was almost equally so.

The characteristics of the diet squad members, which are of interest in this connection, are given in table 1.

After the 16th day, there were 5 men and 5 women until the 42nd day, i.e., through period VII, then 4 women and 5 men through period VIII, 4 of each sex through period IX, 4 women and 3 men through period XI, and thereafter 4 women and 5 men (see tables 3 and 4).

All members fell within the age limits of 21 to 30 years, inclusive. The weights given are the means of 2 weights obtained on the 2 days when each person's basal metabolism was determined — a period extending from August 8 to 28. The surface areas calculated from the formula of DuBois and DuBois are based on these mean weights and the original net heights. Likewise the caloric intakes are taken from the records of consumption at these times. With 1 exception (no. 11) there was no change in caloric intake during the last 4 periods. Adjustments of 50 to 100 cal. (rarely more; and always affecting the carbohydrate components only) were made from time to time as necessary to keep the subjects as nearly as possible at constant weight. The severely hot, humid weather prevailing in Rochester during a good share

⁴Their hemoglobin values, very kindly determined by Dr. E. E. Garber, were found to be within the normal range.

of the summer made it difficult to arrive at the exact requirements early or to maintain them evenly.

The last column in table 1 reflects also, to a considerable degree, the effect of warm unstimulating weather. The average of 32.5 cal. per square meter per hour for the women represents a deficit of 9.7% from the average for women between 20 and 30 years given by Boothby, Dunn and Berkson ('36), and the average of 35.9 a deficit of exactly 10% from the same standard for men within the same ages.

TABLE 1
The diet squad

NO.	SUBJECT	AGE	SEX	HEIGHT	NET WT.	SURFACE AREA	CALORIE INTAKE	BASAL MET. CAL/M ² /HR.
				<i>cm</i>	<i>kg</i>	<i>m²</i>	<i>Per day</i>	
1	D.C. ¹	28	F	171	79.4	1.91	2600	29.4
2	E.R. ¹	21	M	175	72.0	1.86	3300	33.5
3	H.M.	27	M	163	54.5	1.57	2800	36.8
4	L.R.	30	M	185	70.3	1.94	3150	35.3
5	J.W. ¹	22	F	166	60.1	1.67	2200	33.2
7 ²	J.M. ¹	24	F	163	55.2	1.58	2650	31.1
8	A.F.	26	F	172	74.0	1.86	3000	35.4
9	W.K. ¹	22	M	181	79.1	1.99	3500	36.0
10	M.S.	26	F	156	54.5	1.53	2750	36.9
11	J.R. ¹	24	M	184	80.1	2.03	3550	34.7
12	M.E. ³	27	M	176	73.0	1.88	3400	36.7
13	F.H. ³	28	M	176	74.1	1.90	3400	38.1
14	H.G. ³	22	F	157	44.9	1.42	2000	29.3
Average for 6 women								32.5
Average for 7 men								35.9

¹ These members served throughout the whole series.

² No. 6 dropped out earliest. The original number assigned each one was maintained in the interest of identification.

³ These members were recruited in period X (see table 3).

The basal diet

The basal diet is illustrated in table 2 by that consumed by subject D.C. for period IX. The distribution of calories, (2.8% from protein, 62.2% from carbohydrate and 35% from fat) is fairly typical of the diets consumed by all sub-

jects when the protein constituent was of high biological value. (Observe the range of average test protein nitrogens ingested from wheat gluten to whole egg powder in figures 1 and 2.)

Butter was of the best creamery product⁵ obtainable in Rochester. The pure fat was separated by melting and filtering in a hot funnel to remove traces of casein. The syrup

TABLE 2

The daily basal diet with egg albumen as the test protein (subject D.C., period IX)

FOOD ITEM	AMOUNT	NITROGEN
		<i>gm</i>
Butter (gm)	27	0.005
Syrup (gm)	75	0.019
Biscuit Mix with arrowroot starch (gm)	194	0.028
Lettuce, iceberg heart leaves (gm)	50	0.059
Carrot (gm)	25	0.019
Pickle (number)	1	0.007
French dressing (tablespoonful)	2	0.004
Orangeade, artificial (gm)	151	0.010
Applesauce (gm)	100	0.034
All vitamins (see text)	..	0.014
Coffee (number of cups)	1	0.047
Coca cola (bottles)	2	0.027
	Total N	0.273
	Less caffeine N	0.074
		0.199
	Egg albumen powder (21.7 gm)	2.66
	Total available food N	2.86
Total calories: 2600.		
Distribution of calories: protein 2.8%, carbohydrate 62.2%, fat 35%.		

consisted of white corn syrup⁶ to which some cane sugar and maple flavoring were added. It alternated with marmalade and pure fruit jellies as sources of flavor, and for spreads on the biscuits or "griddle cakes" made from the same mix. Arrowroot starch was chosen as the main component because of its very low nitrogen content. The actual composition of

⁵ "Land-O'-Lakes" brand.

⁶ "Karo" brand.

the mix varied but little from the following formula: Arrow-root biscuits — each biscuit (or pancake or cookie depending upon amount of H_2O added), 18 gm; arrowroot starch, 8 gm; sugar, 8 gm; hydrogenated cottonseed oil⁷, $1\frac{1}{2}$ gm; baking powder, $\frac{1}{2}$ gm; salt mix. The lettuce, grated carrot, with 3 seedless raisins, added for aesthetic effect, and a single sweet-sour pickle were served as a salad with the French dressing, composed of the purest cider vinegar and corn oil.⁸

The artificial orangeade was a mixture of white corn syrup, cane sugar, tartaric and citric acids in small amounts, orange extract flavoring and orange vegetable coloring. The apple sauce⁹ had a carbohydrate content of 18%. There was some variation in the nitrogen content, denoting perhaps different mixtures of apples in different lots of 10-pound cans received.

Two different mixtures of B-complex vitamins designated *a* and *b* were used — one designed to supplement the vitamins contained in the natural food items, and the other designed not only to supplement, but also to stimulate and sustain appetite as the long series of periods wore on toward the end. These two expressed in milligrams per man per day are shown below.

	<i>a</i>	<i>b</i>
Riboflavin	0.0 mg	3.47 mg
Niacin	15.0 mg	20.5 mg
Para-aminobenzoic acid	6.3 mg	8.8 mg
Ca pantothenate	3.7 mg	6.4 mg
Pyridoxine · HCl	3.1 mg	4.0 mg
Thiamine · HCl	1.5 mg	2.2 mg
Inositol	100.0 mg	120.0 mg

All these constituents were dissolved in hot water in such quantity as to give the allotted proportions of the mixture *a* in 1 ml of solution when added to the artificial orangeade once a day. Riboflavin and choline were dissolved in 40% alcohol and dispensed in sufficient concentration to provide each subject 2.25 mg of the former and 15 mg of the latter in 1 ml

⁷ Crisco.

⁸ "Mazola" brand.

⁹ "Blue Boy" brand made by the Haxton Co. of Oakfield, N. Y.

of solution, likewise added once a day to the orangeade, but taken at a different meal than the mixture *a*. In the *b* mixture, riboflavin was included, the whole being dissolved in water, to which about one-tenth of the volume of 95% alcohol was added. The solution was brought to boiling temperature, then cooled and bottled. Choline was dissolved separately in water in sufficient quantity to provide 48 mg of the substance in 1 ml of solution for each person, and always dispensed at a different meal than the *b* mixture.

One ascorbic acid tablet containing 100 mg and at first 1 capsule containing 1000 units of vitamin D and 5000 units of vitamin A were dispensed to each subject daily. Later the same unitage for the AD capsule was supplied by cod liver oil tablets,¹⁰ thus avoiding nitrogen from the gelatin capsule.

The salt mixture employed in this study was, with very slight modifications, the same as that employed by Murlin, Edwards, Hawley and Clark ('46) and described on p. 535 of that communication. Coffee or tea was served at any meal desired, but was limited to 2 cups daily. It was prepared from soluble powder.¹¹ Tea bags were used in making tea, 1 bag to 2 cups. On account of their low N content, certain carbonated beverages¹² were permitted up to 2 bottles daily. The nitrogen in all of these beverages, called for convenience "cafein nitrogen," was determined frequently and always deducted from the nitrogen intake as having no food value. The corresponding amount for each subject was deducted from the urinary nitrogen, because it is usually excreted quantitatively.

The total nitrogen of the basal diet, less the cafein nitrogen in the case illustrated, was just short of 0.2 gm daily, but the average for the 8 members in this period was 0.184 gm or 5.7% of the total N. Averages for all the periods were: III, 0.29; IV, 0.35; V, 0.28; VI, 0.33; VII, 0.26; VIII, 0.23; IX, 0.18;

¹⁰ From the White Laboratories, Newark, N. J.

¹¹ "Nescafe."

¹² "Coca Cola" and "Canada Dry" ginger ale.

X, 0.23; XI, 0.20; XII, 0.23; XIII, 0.23; XIV, 0.20; and XV, 0.21. The percentages of total food nitrogen ranged from 9% in period IV to 4.0% in period XIV.

Nitrogen determinations of all constituents of the basal diet as well as of the proteins fed were made in every period; but the 3 items making up the salad were for the most part homogenized together in the Waring blender and determined as one to save time. Occasionally, the sum of the individual analyses was checked by taking aliquots of all items of a day's dietary, homogenizing them together and making but 1 analysis. The agreement was invariably quite close.

All analyses of food materials as well as of the urines and feces were made by the Macro-Kjeldahl method, employing sodium sulphate and copper sulphate in the proportion of 9:1 as catalyst and saturated boric acid, to which brom cresol green and methyl red in the proportion of 1:10 were added, in the receiving bottle for distillation.

Urines were collected in 24-hour periods for all days of the period but were analyzed, as a rule, on only the last 3 days. Feces were separated in periods by carmine or ferric oxide markers. Until the marker was obtained they were passed into white enameled pails; thereafter, directly into the 1-gallon "candy" collecting jars containing a few hundred ml of 5% sulfuric acid to which 10% of copper sulfate had been added. Special commodes designed to hold the jar or pail as well as a urine bottle were used.

Preparation of the food ("test" proteins)

In addition to the above-mentioned food items and their method of handling which comprised the "non protein" fraction of the diet, even greater precautions were necessary in the handling of the test proteins.

The fresh eggs were obtained from the same flock of birds fed on a constant diet. They were broken and approximately measured as to quantity (assuming 50 gm per egg) into the bowl of the electric mixer. They were then beaten at low speed

for 10 minutes, strained into a pitcher through a fine strainer and the desired amount poured with repeated stirrings directly into weighed beakers, on a balance. They were cooked by 3 methods. In periods 1, 2 and 7 they were scrambled or made into omelet on the stove top. In period 12 they were served as baked custard. They were so cooked in this period as to be in exact conformity with the other test proteins — in pyrex dishes placed in a pan of water and baked for 40 minutes at 350°F. In all periods where fresh egg was served 6 gm of the prescribed amount was added to the biscuits.

In general, the test protein was added to the biscuit mix and baked in pyrex dishes in the manner of the custard cooking. The biscuits were served to the subjects in the baking dish. Spatulas and rubber policemen, in addition to rinsing with water at the end, assured a quantitative transfer of protein.

It was not feasible, however, to add all of the egg albumen to the mix because the biscuits became too brittle and hard. A portion of this protein, therefore, was baked after mixing with water and served as baked egg white custard. Since it was impossible to prepare palatable biscuits incorporating the beef powder, some of it was served in soup and the remainder as a meat patty by the addition of different amounts of water. The cooking was done in a pan of water in the usual manner.

The raw casein obviously was not cooked. It was all mixed into a paste with water and eaten directly from the container with a spoon.

Table 3 exhibits the order of feeding the several test proteins, and the position of the fresh whole-egg and no-protein periods immediately following. Preparing the subjects by a high-value protein, from which there is little waste, for the no-protein period has a standardizing value which has been discussed by Murlin, Edwards, Hawley and Clark ('46). The table also gives the number of days for each period, influenced to some extent by the acceptability of the protein; the average nitrogen balance for the last 2 or 3 days of each; and

the number of subjects contributing to the balance. The reason for variation in the number of subjects has already been discussed.

The first no-protein period, it will be observed, ran for 7 days. Preceding it were 15 days — 3 before collections were started — on whole fresh egg. This protein was fed for the

TABLE 3
Order of feeding periods

NO.	PROTEIN	DAYS	AVERAGE N BALANCE	NUMBER OF SUBJECTS
			<i>gm</i>	
I	Fresh whole egg, begun June 6	6	— 0.28	11
II	Fresh whole egg, begun June 12	6	— 1.22	11
III	No protein	7	— 2.40	10
IV	1st casein powder	4	— 0.06	10
V	2nd casein powder (slightly higher N)	4	+ 0.36	10
VI	3rd raw casein	4	— 0.49	8
VII	Fresh whole egg	7	+ 0.13	10
VIII	No protein	5	— 2.68	8
IX	Egg albumen powder	5	— 0.15	8
X	1st whole egg powder	5	— 0.46	7 + 3 ¹
XI	2nd whole egg powder (lower N)	5	— 0.61	7 + 3
XII	Fresh whole egg (No protein for 3 new subjects)	6	+ 1.04	6 + 3
XIII	Wheat gluten	5	— 2.58	9
XIV	Peanut flour	6	— 0.14	9
XV	Beef powder, ended Aug. 30	6	+ 0.33	9

¹ Three new subjects joined the squad during this period but subsisted on fresh whole egg until the no-protein period for them (per. XII).

first 9 days at a level of 5% of the total calories, and in period II at 3%. The protein reserve of the subject thus was brought down gradually to near the very low point of the no-protein period. The lowest urinary nitrogen of the last 2 days was accepted as that for the endogenous level of excretion. The average for the 10 subjects was 2.01 gm. The average for 8 subjects in period VIII, which ran for only 5 days, was 1.97.

Including the 3 subjects whose no-protein ingestion coincided with that for period XII, the average for 9 persons for the last 3 periods was 1.86 gm (see line h of table 4). The relation of the individual endogenous urinary N to basal metabolism will be found in table 5.

RESULTS

The biological values

The data for estimation of the absorbed and the retained nitrogen values, from which the B.V.'s are calculated, are shown for the several proteins side by side in the order of feeding (table 4).

Three periods were devoted to the dry casein product. The method of preparation, as received from the coördinating committee, will be found in the addendum. It proved to be a very harsh, insipid powder and it required some experimentation, before the study started, to arrive at a satisfactory method of preparation, as has been related. The second period differs from the first in providing an average of almost 1 additional gram of ingested nitrogen for the squad of 10. This is the only reason assignable for the 0.3 gm higher average fecal excretion in this period. Obviously it is proportionally higher as shown by the lower percentage digestibility. The first period produced a very small fecal waste from the protein as measured by the excess over the no-protein period. A difference between the 2 periods of 0.67 gm in the calculated absorbed nitrogen resulted in only 0.26 mg more nitrogen in the urine. The no-protein urinary nitrogens being the same in the 2 periods, the waste nitrogens differ by the same amount, and these subtracted from the absorbed give retained nitrogens representing percentages of the absorbed (B.V.'s) that differ by only 1 unit. The raw casein was fed at a level midway between the 2 cooked casein periods. It proved not only less digestible but also less well utilized beyond the alimentary tract. The result was a biological value

TABLE 4
Biological values of 6 proteins
 (Gm. N — Average of all members of squad)

	CASEIN 1	CASEIN 2	CASEIN RAW	EGG ALBUMEN	POWDERED WHOLE EGG	WHEAT GLUTEN	PEANUT	BEEF POWDER
(a) Test protein fecal N in gm	0.76	1.06	1.10	0.89	0.95	0.81	1.07	1.14
(b) No-protein fecal N in gm	0.72	0.72	0.72	0.94	0.94	1.01	1.01	1.01
(c) Test protein waste fecal N in gm (a-b)	0.04	0.34	0.38	— 0.05	0.01	— 0.20	0.06	0.13
(d) Test protein eaten N in gm	3.91	4.88	4.34	3.23	2.44	5.33	4.90	4.96
(e) Absorbed N (d-c)	3.87	4.54	3.96	3.28	2.43	5.53	4.84	4.68
(f) True digestibility $\frac{(e \times 100)}{d}$ in %	98	93	91	101	98	104	99	97
(g) Test protein urinary N (gm)	3.20	3.46	3.74	2.25	2.09	5.07	3.97	3.38
(h) No-protein urinary N (gm)	2.01	2.01	1.99	1.97	1.95	1.86	1.86	1.86
(i) Test protein waste urinary N in gm (g-h)	1.19	1.45	1.75	0.29	0.14	3.21	2.11	1.52
(j) Retained N (e-i)	2.68	3.09	2.21	2.99	2.29	2.32	2.73	3.16
(k) Biol. val. $\frac{(j \times 100)}{e}$	69	68	56	91	94	42	56	67
Number of subjects	10 5 ♂ 5 ♀	10 5 ♂ 5 ♀	9 5 ♂ 4 ♀	8 4 ♂ 4 ♀	7 3 ♂ 4 ♀	9 5 ♂ 4 ♀	9 5 ♂ 4 ♀	9 5 ♂ 4 ♀

of only 56 as compared with 69 and 68 for the 2 previous periods.

These results are shown graphically in figure 2 for the first cooked casein and the raw casein. Note the large amount of fecal and urinary waste nitrogens for the latter as indicated by the bracket and double arrow, respectively. Compare with egg albumen and whole egg on the same chart.

There is little more that needs to be said in explanation of table 4 or figures 1 and 2.

The first whole egg powder period (X) produced an average B.V. for the 7 subjects of only 75. It has been observed in other studies in this laboratory that a value like this is obtained once in a while, and it is believed such values are correct for the time and circumstances. But until an explanation can be given for these exceptional values they will be omitted. The one shown in table 4 and figure 2 is near the average of the many determinations in this laboratory on whole fresh egg. (Note 2 higher values for the fresh egg in figure 1 of the paper by Murlin et al. ['48].)

A question might be raised regarding the use of a negative difference between no-protein and food fecal nitrogens (table 4, egg albumen and wheat gluten) indicating higher than 100% digestibility and resulting in more absorbed than ingested nitrogen. However, it is not surprising that these 2 proteins should be more digestible than the small amount of protein contained in the no-protein diet, or that there should be less residual nitrogen from the intestinal secretions with highly digestible proteins. For these reasons, such differences have been used algebraically in this study, as shown.

Endogenous nitrogen of the urine

Since the work of Terroine and Sorg-Matter ('27) and of Smuts ('35), it has been maintained that mammalian species show a definite relationship between endogenous urine nitrogen and basal metabolism (Bricker, Mitchell and Kinsman, '45; Bricker and Mitchell, '47) such that 2.2 to 1.43 mg of the former correspond to 1 basal Calorie. In the 1946 study

Biological Values

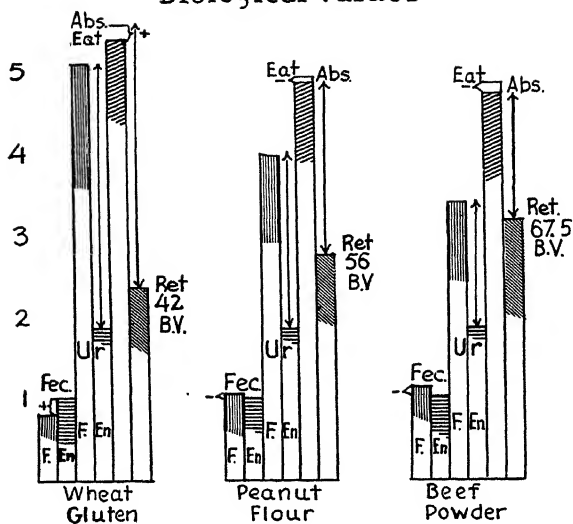


Figure 1

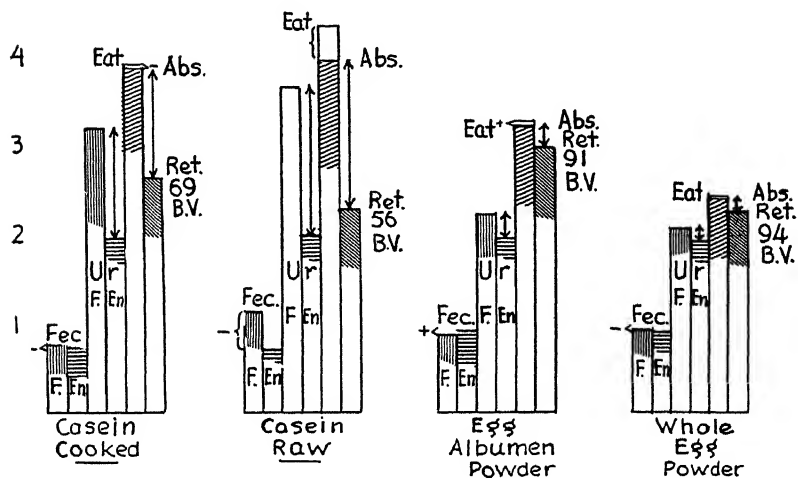


Figure 2

In each group of 6 bars:

1. The first pair of bars on the left refer to fecal nitrogen; the one marked "En" refers to the no-protein period and the one marked "F" refers to period in which the test protein was fed.
2. The center pair of bars represent the urinary excretion of nitrogen in protein and no-protein periods, respectively. The arrows represent waste nitrogen in excess of endogenous excretion.
3. The next to the last bar represents the nitrogen ingested and absorbed (the latter by diagonal hatching).
4. The last bar on the right represents the retained nitrogen. The figure alongside is the computed biological value (B.V.).

of endogenous nitrogen (Murlin, Edwards, Hawley and Clark), this ratio was found to be lower in 5 male subjects. Confirmation of this is seen in table 5, notwithstanding that the basal metabolisms of the 13 subjects averaged 10% below normal for their ages.

It would not be profitable at this time to compare the results regarding biological values with those in the literature. A

TABLE 5
Relation of endogenous N of urine to basal metabolism

SUBJECT	SEX	ENDOGENOUS URINARY N	BASAL METABOLISM	RATIO OF MG TO CAL.
		<i>mg/24 hr.</i>	<i>Cal./24 hr.</i>	
1	F	1910	1348	1.42
2	M	1960	1497	1.31
3	M	1760	1387	1.27
4	M	1990	1646	1.21
5	F	1510	1330	1.13
7	F	1310	1180	1.11
8	F	1800	1580	1.14
9	M	2450	1718	1.43
10	F	1570	1356	1.11
11	M	2610	1690	1.54
12	M	2075	1656	1.25
13	M	2170	1740	1.25
14	F	1240	998	1.24
Average of 6 women				1.19
Average of 7 men				1.32

comparison of all the new results with older ones should be made. So far as is known, this is the first of the several coöperative studies to be offered for publication. It will therefore be an inviting target, not to say a vulnerable one.

ADDENDUM

Egg albumen. The liquid egg white, either fresh or rapidly defrosted, is warmed to a specific temperature and inoculated with a culture to hasten fermentation. It is then allowed to ferment until all of the natural sugar is completely eliminated. After fermentation is complete, the liquid is chilled to stop fermentation.

It is held at reduced temperature until pumped to the dryer. The material is then converted to an aerated foam and dried continuously at an inlet air temperature not to exceed 150°F. (65.5°C.). The drying time is approximately 40 minutes. The product comes from the dryer in a very light fluffy form and is ground by merely rubbing it through a screen. This is, of course, done mechanically. The result is a product dried quickly at comparatively low temperatures, a product each particle of which has a very irregular surface which aids reconstitution with water.

Beef-benzol extracted muscle. Utility beef was used. The meat was trimmed as much as possible from fat and gristle and then ground. It was next heated to 180°F. (82.2°C.), spread on trays and dried in vacuum from the frozen state. It was then defatted with benzol and again dried in vacuum at low temperature to remove all solvent. The product was powdered sufficiently for incorporation into diets.

Whole egg. Frozen whole fresh eggs were used. They were desiccated and defatted by ethylene dichloride at temperatures below 70°C. Ethylene dichloride was the only reagent that came into contact with the product and it has been shown by Mitchell and others, who have examined other products, that this solvent in no way denatures the protein.

Peanut flour. Prepared by the McMath-Howard process, resulting in a partially defatted flour. The approximate analysis (in per cent) is protein 58.98, fat 9.69, N free extract 20.51, ash 3.84, crude fiber 2.54, moisture 4.44, phosphorus 0.565, calcium 0.065, magnesium 0.365 and iron 0.010. The vitamins (in $\mu\text{g/gm}$) were thiamine 6, riboflavin 3 and niacin 189.

Wheat gum gluten. Wheat flour agene-treated, of about 12% protein content, was selected. Cold water was added to form a dough in order to hydrate the gluten. More cold water was added to remove the starch leaving behind the gummy gluten. Five to 6 washings with water up to 90°F. were followed by 1 or 2 washings with water up to 170°F., the number of washings and the temperature depending upon the difficulty encountered in removing all the starch without losing the gummy properties of gluten. When the protein content was brought up to 80% on a dry basis, the material was dried on trays in a vacuum at 130°F. Each batch was specially handled in order to avoid denaturation of the protein (loss of gummy properties) as much as possible. Each batch varied from 4 to 5,000 pounds. It was not defatted. Actual temperature of gluten mass probably never exceeded 140°F., even when washed with water at 170°F.

Casein. The casein was prepared from a crude acid precipitated product. Purification consisted of repeated solution and reprecipita-

tion as well as thorough washing with dilute aqueous acid and salt solutions at temperatures of 40–55°C. No organic solvents were used. After drying in a stream of air at 60–70°C., the casein was ground in a hammer mill until 97% passed a 60-mesh sieve.

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CREATININE NITROGEN PERCENTAGE AS A CHECK ON THE BIOLOGICAL VALUES OF PROTEINS

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ONE FIGURE

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For several years past this laboratory has systematically used the creatinine nitrogen of the urine as a check on quantitative collections. Ever since Folin's ('05) famous paper on his new methods for urine analysis it has been known to be the most constant constituent on a creatine and creatinine free diet. In the course of the study described in the previous paper (Hawley, et al., '48) it had been observed that the creatinine nitrogen excretion expressed as milligrams daily was frequently very constant, not only for a single individual but also as an average of all squad members. Also that when expressed as percentage of the total nitrogen, there was a marked difference between high and low value proteins. This led to a study of the correlation between these 2 values not only from the most recent data available to us but also from earlier studies in this laboratory.

There are good reasons for expecting a high correlation between any constant component as a percentage of total nitrogen and the amount retained. Creatinine is without doubt the most typical endogenous product. Since biological values are calculated by subtracting the endogenous urinary nitrogen from the total urinary nitrogen after feeding the test protein,

the most genuinely endogenous constituent will, of necessity, vary in percentage of the total nitrogen inversely as this total nitrogen rises and falls with different kinds of food proteins. If the waste nitrogen of the food is high, this denotes a low retention and therefore low B.V., and produces a low creatinine N percentage; and vice versa.

A list of 14 periods were found, 9 from the study described in the preceding paper and 5 from the data of squads 8 and 9 of the paper by Murlin, Edwards, Hawley and Clark ('46, table 2) where the endogenous urinary nitrogens were unimpeachable and the biological values satisfactory. The correlation coefficient between the deviations from the mean values for biological value and for percentage of creatinine N in the urine was found to be 0.972. When the several means for the 2 variables obtained from the 14 periods for determination of biological values were plotted, the distribution seen in figure 1 was obtained. The ideal regression line was fitted to the data by the method of least squares or errors of estimate by the equation $E = \bar{y} + \frac{S_{xy}}{S_x^2} (X - \bar{x})$, according to Snedecor ('37). E is the estimate of the ordinate (biological value) for any chosen value of the abscissa X (creatinine percentage), \bar{x} is the mean of the determined values, and \bar{y} is the mean of the ordinates. S_{xy} is the sum of the products of the deviations from the means, and S_x^2 is the sum of the squares of the deviations from the mean creatinine percentage.

The position of the conjunction of the 2 means, \bar{x} and \bar{y} , is at the central X . The value of E for 16.4% creatinine nitrogen is found by the computation to be at the position on the line of the lower X , and the value of E for 21% creatinine N is at the position of the upper X . The regression line drawn through these 3 points describes the average rate of change in E for a unit change in X .

From the first 4 points located on the plotting, it was possible to predict approximately the biological value of wheat gluten from the average creatinine nitrogen percentages. From the average of these percentages for the first day of analysis when peanut was the test protein, its biological value

was predicted quite accurately, as determined later when all analyses were at hand. It will, of course, require many more data before the relationship can be established with finality.

The conditions which will produce still more constant creatinine excretions have yet to be worked out by much experimentation. The photoelectric colorimeter by which all

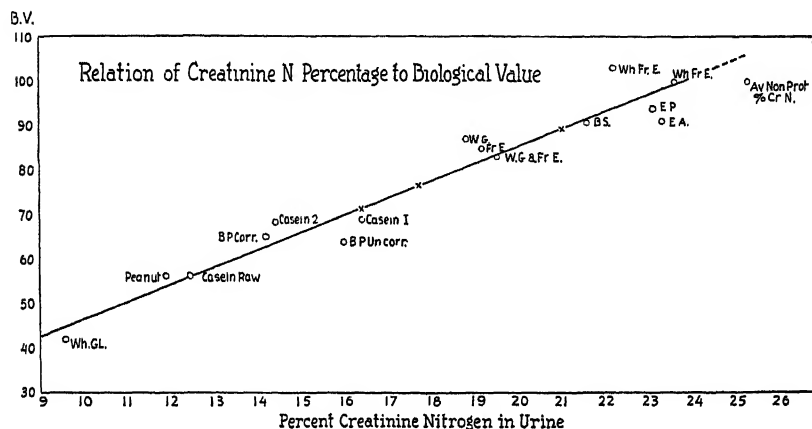


Fig. 1 Wh.G.L. denotes wheat gluten, B.P. corr., and B.P. uncorr., beef protein corrected and uncorrected for the excess creatinine nitrogen in the urine of the beef period over that of the peanut period which just preceded it. The 3 casein periods of the tests described in the current study are indicated. E.P., E.A. and Wh.Fr.E. (lower) indicate the coordinate points for whole egg powder, egg albumen powder and for whole fresh egg protein of periods XI, IX and VII. W.G., Fr.E., and W.G. & Fr.E., are points taken from the values found for wheat germ, fresh whole egg, and wheat germ 50% and fresh whole egg 50% — all from the data of squad 9 of the 1946 study. Wh.Fr.E. (upper value B.V. 104) and B.S. are for fresh whole egg and beef steak as found from squad 8 in the same study. B.P. uncorr. is not counted in the correlation.

creatinine determinations have been made in this laboratory for many years, is so much more dependable than the old Duboseq method that older values would scarcely be acceptable.

DuBos and Miller ('37) have shown by their specific enzymatic determination that creatinine of normal and even of nephritic urines constitutes nearly 100% of the chromogenic

substance contained. Therefore, unless some kind of stable artificial coloring matter from the food should occur in the urine, the excretion through normal kidneys certainly would reflect the rate of endogenous metabolism from a diet containing no creatine or creatinine.

If, as in the case of the beef powder (B.P. on the graph), the creatinine content of the food can be determined, a correction can be applied. The powder was extracted with hot water until no chromogenic reaction was obtained by the alkaline picrate reagent. The result was an average of 149 mg for the average amount of the powder ingested. Apparently this was not quite all excreted for the difference between the

TABLE 1

*Average creatinine excretion of 6 subjects who were longest on the diets.
All values are in milligrams of creatinine N daily*

SUBJECT NO.	SEX	AVERAGE OF FIRST 10 DAYS	AVERAGE OF NEXT 20 DAYS	AVERAGE OF LAST 15 DAYS	VARIABILITY OVER ALL IN %
1	F	505	458	451	466 \pm 27 \pm 5.8
2	M	553	537	533	535 \pm 26 \pm 4.8
5	F	396	359	342	363 \pm 21 \pm 5.8
7	F	390	360	347	363 \pm 17 \pm 4.7
9	M	642	608	605	620 \pm 20 \pm 3.2
11	M	659	651	616	639 \pm 32 \pm 5.0

creatinine of the beef period and of the peanut period was only 114 mg. Peanut gave no chromogenic reaction. Consequently, the correction exhibited in the graph was based on the average difference for all the 9 members of the squad.

The variability in creatinine nitrogen, as shown by the 6 members who served throughout the entire series of experiments, is shown in table 1. There was, with 1 exception, a rather large decrease from the first 10 days and the next 20 days but (again the same exception) only a slight change to the last 15 days.

The variability for the entire 45 days on which urinary analyses were made is only from 3.2% for subject 9 to 5.8%

for subjects 1 and 5. Such variations do not invalidate the correlation shown in the graph.

It is of interest to note that the creatinine percentage of the total N on the no-protein diets used in this laboratory is 25.3, the mean of 39 determinations. This value is placed at the 100% line in the graph, not to indicate a biological value, for that obviously is not possible for the very small nitrogen intake, but to call attention to the fact that some constituent of the diet, possibly carbohydrate, exercises a sparing action on creatinine. The regression line is dotted to the position which a B.V. corresponding to 25.3% would occupy. Without doubt, the percentage would be higher in starvation.

It would seem that the biological value of any mixed diet could be obtained approximately by feeding it at a level low enough to obtain a minus N balance, and determining the creatinine nitrogen percentage. This possibility is now under investigation.

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FOOD AND WATER ECONOMY OF THE YOUNG RAT DURING CHRONIC STARVATION AND RECOVERY

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TWO FIGURES

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Only a few scattered and unrelated conclusions have been made pertaining to the alterations in food utilization and water consumption during chronic starvation and recovery. Thompson and Mendel ('18) have shown that in mice the weight gained during refeeding of undernourished and stunted animals was greater in proportion to the food eaten than that gained by the controls. Keys et al. ('46) found that in adult men the weight recovery was greatest on high caloric diets. It is stated by Morgulis ('23) that the gross gain in weight may exceed the quantity of food consumed due to the fact that during re-alimentation the organism retains much water. Benedict ('07) and Keys et al. ('46) also found that water is retained during starvation, and noted in this connection an increase in water intake and water excretion.

The studies made in this experiment to ascertain the influence of chronic starvation and recovery on food and water economy were approached in the following ways: (a) by determining the amount of food and water required each day to maintain a constant body weight during chronic starvation, (b) by observing the course of recovery of body weight both as to the amount of food and water consumed and the time required, and (c) by determining the degree of absorption of

food materials in chronically starved rats as compared to that in normal rats.

METHODS

The investigations made in this work were arranged in 2 separate experiments. In both experiments young male albino rats, 30 days of age and about 50 gm in weight, were employed in groups of 50, each group constituting an experimental unit. The rats were placed in individual cages and were underfed by restriction to a constant daily body weight ration of a qualitatively balanced diet. The synthetic stock food employed is shown in table 1.

TABLE 1
Composition of synthetic stock ration in amount per gram

Protein	240.0 mg	Thiamine	0.005 mg
Carbohydrate	450.0 mg	Pantothenic acid	0.012 mg
Fat	45.0 mg	Niacin	0.0056 mg
Fiber	40.0 mg	Riboflavin	4.0 μ g
Ash	100.0 mg	Vitamin D	60.0 chick units
Water	100.0 mg	Vitamin A	600.0 U.S.P. units
Salt	2.5 mg	Vitamins C, E and K	present in
Iron oxide	2.5 mg	unmeasured amounts	

Adjustments of the amount of food allowed daily were made with each rat according to the loss or gain in body weight. At the end of the selected periods of underfeeding the animals were given full and adequate amounts of the above ration for 2 different periods of time. In experiment I, 50 rats were underfed for 30 days and refed for 35 days; in experiment II, 50 rats were underfed for 90 days and refed for 60 days. In addition to these groups of refed rats, a group of fully fed controls was observed during the recovery period of experiment I; the animals of this normal group were of the same size as the experimental animals at the end of the starvation period.

Daily food consumption during recovery was determined by placing a given quantity in the cages and weighing the dry residue 24 hours later. Water consumption was determined by

recording the number of times cage bottles were filled. Wax lines were marked on the bottles so that refilling represented a certain number of milliliters. The animals were weighed daily in experiment I and weekly in experiment II. All weights were taken at the same time each day and on the same day each week.

There was reason to believe that food-feces ratios would throw light on the problem of food economy. It is conceivable, for instance, that an increase in the ability of the digestive system to absorb usable material from ingested food could contribute to body weight gain. Ficker ('06) speaks of the increased permeability of the epithelial lining of the digestive tract to microorganisms as a result of starvation. Brooks, Marine and Lambert ('46) employed food-feces ratios with success in determining the efficiency of digestive functions in experimental obesity. Methods similar to those employed by these workers were used in this experiment. Food intake was determined for a period of 5 days by weighing the dry residue in the cages of 10 control rats and in those of 10 experimental rats which had been starved for 60 days. The dry weight of the feces was determined by collecting and desiccating the feces from both the control and experimental groups over this same period.

RESULTS AND DISCUSSION

The average daily food rations necessary to maintain a constant body weight during starvation are shown in figure 1. Although all rats in the preliminary experiments were allowed an identical daily ration, there was considerable variation in the ability to withstand sudden caloric restriction. Rats that survived the first few days of restricted food intake lived throughout the starvation periods. Gradual reduction in the daily ration in early starvation eliminated mortalities and made it possible later to maintain all animals on the same amount of food. Mortalities which nearly always occurred at the beginning of starvation in the preliminary experiments were eliminated entirely in the final studies by individual

food and body weight adjustments. The animals in chronic starvation required progressively less food to maintain body weight as the underfeeding was continued. These results may be viewed as an adaptation or acclimatization. The probable mechanism here is twofold: (a) the increased absorption efficiency of the digestive tract of the starved rats, as appears below in the results of this experiment and (b) the reduction in metabolic rate which resulted from starvation (Quimby and Phillips, '47). Both of these effects would improve the food economy of the animal, the former actually providing

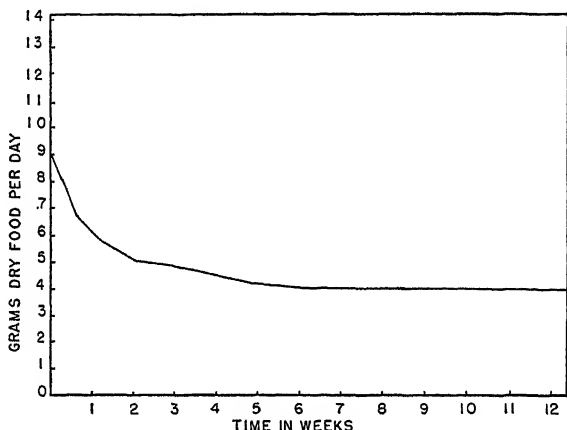


Fig. 1 The amount of food required daily during chronic starvation to maintain a constant body weight in young rats. The curve represents an average of 50 animals.

more energy for the tissues, the latter reducing the basal energy demand.

The average total water intake, including that consumed with the food, for the starved and fully fed normal rats during a 30-day period is presented in table 2. The amount of dry food consumed by these 2 groups over this same period and the food-water ratio are also shown. These data show that although there was greater water intake in proportion to the amount of food consumed on the part of the starved rats, the total water consumption was not great. This finding

stands in contrast to (1) that of Benedict ('07) who observed that starving human subjects drank enormous volumes of water, and (2) that reported by Keys et al. ('46) who noted that semi-starved men more than doubled their water intake. It appears, therefore, that the water retention noted by Benedict in starving humans and the excessive diuresis observed by Keys et al. in semi-starved men were not typical of the chronically starved young rats of this investigation.

The relationship between the weight of dry food consumed and the weight of dried feces is expressed in table 2. These figures show that there was greater food absorption in the starved rats than in those which were fully fed. This fact suggests either a decrease in peristalsis or an increase in the permeability of the epithelial lining of the digestive tract.

TABLE 2

Food-water and food-feces ratios of chronically starved and fully fed rats

EXPERIMENTAL GROUP	FOOD CONSUMPTION	WATER INTAKE	FOOD-WATER RATIO	WEIGHT OF DRY FECES PER DAY	WEIGHT OF DRY FOOD PER DAY	FOOD-FECES RATIO
	<i>gm</i>	<i>ml</i>		<i>gm</i>	<i>gm</i>	
Starved	150	373	1: 2.5	1.14	4.5	1: .25
Fully fed	520	984	1: 1.9	5.20	17.1	1: .34

The daily food consumption of starved rats during the first 20 days of recovery as compared to that of normal rats of the same size is presented in figure 2. Anorexia did not develop in any of the starved animals; food upon re-alimentation was accepted with vigor. However, during the first 10 days of recovery feeding the rats which had been chronically starved exhibited less appetite than normal rats of the same size. The only exception to this occurred on the very first day that food was allowed ad libitum. After the 12th day of re-alimentation the rats recovering from chronic starvation had a greater appetite than the controls and ultimately accumulated a greater total food consumption (table 4 and fig. 2). No deleterious effects of the ingestion of large amounts of food on this first day of recovery were noted in these rats,

such as were observed by Benedict ('07) and Keys et al. ('46) in humans. The decreased food intake on the second day may indicate some reaction or illness of the animal, or may have been simply an appetite reduction due to the indulgence of the first day. The latter is the more likely explanation since it was observed that in both normal and refed rats there was a tendency for food consumption to alternate between high and low on successive days.

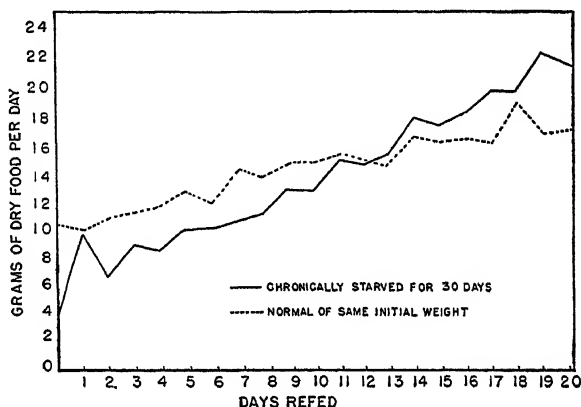


Fig.2 Comparison of daily food consumption of refed starved rats with normal rats of the same initial weight. Each line on the graph represents the average of 10 rats.

The relationship between food consumption and weight gain from the 60-day recovery period of experiment II is summarized in table 3. It can be seen from these data that the greater caloric intake results in an increased weight recovery. Similarly Keys et al. ('46) noted that the weight recovery in adult men who had been chronically starved was greatest on high caloric diets. A gross gain in weight exceeding the weight of consumed food, as noted by Morgulis ('23) on humans, was not observed in this investigation with rats.

The effects of re-alimentation on food consumption, food utilization, weight gain, and water intake are summarized in table 4, together with similar data for the controls. Upon

refeeding, the rats which had been starved exhibited a remarkably rapid rate of growth. This is shown by the data for the rats of experiment I who after 35 days of refeeding had an average weight of 246 gm compared with the normal rats of

TABLE 3

Food consumption, weight gain, and food utilization during recovery from chronic starvation

NUMBER OF ANIMALS	AVERAGE TOTAL FOOD CONSUMPTION	AVERAGE TOTAL WEIGHT GAIN	PER CENT FOOD UTILIZATION ¹
	gm	gm	
12	808.3 (714-859)	174.4 (132-200)	21.4
16	959.6 (905-1059)	216.7 (204-239)	21.5
8	1218.7 (1105-1346)	271.0 (251-289)	22.2

¹ Grams increase in weight
Grams of food consumed

TABLE 4

Weight gain, food consumption, food utilization and water intake of normal and refed rats

NO. OF RATS IN GROUP	INITIAL WEIGHT	FINAL WEIGHT	WEIGHT GAIN	GAIN	RE-COVERY (OF NORMAL)	FOOD CON-SUMP-TION	PER CENT OF FOOD UTILI-ZATION ¹	WATER INTAKE
	gm	gm	gm	%	%	gm		ml
Experiment I — Chronically starved for 30 days, and refed for 35 days								
50	58.3	246.1	187.9	321.8	83.7	633.1	29.9	852.8
Fully fed control, of same initial weight as experiment I								
10	60.5	195.2	134.7	222.2	63.3	576.0	23.4	1151.0
Experiment II — Chronically starved for 90 days and refed for 60 days								
50	60.3	267.1	206.7	340.8	75.4	965.7	21.3	1834.0

¹ Grams increase in weight
Grams of food consumed

the same initial weight whose average weight after 35 days of refeeding was only 191 gm. This stimulation of growth by starvation may be further emphasized by comparison with the growth rate found by Bryan and Gaiser ('32) in normal rats fed a special growth ration. Fed on what they call

"Mendel's special growth diet" but which is really a ration devised by Smith and Bing ('28), the normal rats observed by these workers required 38 days to grow from 60 to 200 gm. The chronically starved rats of this present experiment required only 22 and 30 days to grow through this identical range of body weight after being starved 30 and 90 days, respectively. Kopec and Latyszewski ('32) interpret this increased growth rate of refed animals as simply indicating a stimulating effect of starvation on growth. Jackson ('25) states that the rapid growth of the body following various periods of inanition may be due to the embryonic condition to which the cells are reduced. The extremely high metabolic rates during recovery found in studies related to the present investigation (Quimby and Phillips, '47) may well support this concept of embryonic cell growth. However, the results of the present study indicate that growth stimulation following starvation may be related to the greater food utilization efficiency which starvation effects. While to some extent the enhanced growth may have been contributed to by slightly greater food consumption, it can be seen in table 3 that the group which had been starved had a food utilization of 29.9% as compared to 23.4% for the controls. This increased food utilization efficiency may have been due to the increased intestinal absorption which was incurred during chronic starvation. Results similar to this have been obtained on mice by Thompson and Mendel ('18) who concluded that the weight gained by the stunted animals in proportion to the food eaten was greater than that gained by the controls.

The recovery of a normal body weight by the stunted animals was, of course, somewhat proportional to the duration of refeeding. Prolonged inanition increased the time for recovery, however. The mean weight of rats starved for 30 days and refed for 35 days was 246 gm, while that of rats starved for 90 days and refed for 35 days was only 220 gm. In other words, after equal periods of refeeding the rats which had been held in a state of chronic inanition for a prolonged period recovered considerably less in body weight than rats which

had been starved for only a short time. It may be concluded, therefore, that while short periods of semi-starvation stimulate growth, prolonged starvation tends to reduce this effect. Whether this difference in growth rate following short and long periods of chronic starvation is a reflection of the differences in ages of the animals, or whether it is a reflection of fundamental tissue changes associated with the malnutrition has not been determined.

SUMMARY

1. There was considerable variation in the ability of the rats to withstand sudden caloric restriction. Gradual reduction in the daily ration in early starvation eliminated mortalities and cognizance of adaptive differences carried out by individual weight losses and food ration adjustments made it possible later to maintain all animals on the same amount of food. The quantity of food necessary to maintain a constant body weight became less as underfeeding was continued.

2. The amount of water consumed by the starved rats was less than that consumed by fully fed rats of the same initial size, but was greater in proportion to the food intake.

3. Chronically starved rats absorbed more material from the food than did normal rats.

4. Anorexia did not develop as a result of chronic starvation, although during early re-alimentation the underfed rats consumed on the average slightly less food than normal rats of the same initial weight. The starved animals when refed accepted food with vigor, and the ingestion of large amounts appeared to have no ill-effects.

5. Animals consuming large amounts of food during re-alimentation exhibited a greater weight gain than those with lesser appetites.

6. Rats underfed for 30 days had a greater growth rate and food efficiency than normal rats of the same size. This effect was not seen in rats of the more prolonged starvation period of 90 days.

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IMPROVING THE NUTRITIVE VALUE OF FLOUR

II. FURTHER STUDIES ON THE EFFECT OF SUPPLEMENTING ENRICHED FLOUR WITH B-COMPLEX VITAMINS AND SOME OBSERVATIONS ON THE USE OF 80% EXTRACTION FLOUR¹

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FOUR FIGURES AND TWO PLATES

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Previous work by Westerman and Hall ('47) has shown that when enriched patent flour was used as the sole source of the B-complex vitamins in the diet of the rat, supplements of certain B vitamins improved the nutritive value of the flour. This has been partially confirmed, particularly in regard to riboflavin, by Guerrant and Fardig ('47).

In the experiments reported by Westerman and Hall ('47) the vitamins were added to the flour in amounts similar to those found in whole wheat in order to replace the vitamins lost in the milling process. However, none of the animals made as good growth as those on the stock diet. This was probably due to an inadequate supply of certain vitamins. In a normal diet the amount of B vitamins in whole wheat, if fed at a 30% or 40% level in the diet, would not be expected to meet the body's needs for these vitamins. However, among the low income groups of the population the diets may be inadequate in meat, milk and vegetables. These materials furnish the best sources of the B vitamins and in many cases are partially replaced by the use of larger amounts of cereals in the

¹ Contribution no. 141 of the Department of Home Economics.

diets. Therefore the question arises as to whether the addition of more of the B vitamins or increasing the amounts of those vitamins already added in the enriching process would prove beneficial. It was decided to conduct a series of experiments, based primarily on the rat's requirements for the B-complex vitamins, using whole wheat and enriched flour supplemented with different amounts of the B vitamins, to determine the effect on growth, maintenance, reproduction and length of life.

In 1946 the government decided that 80% extraction flour would be used for a period of time in this country. This was done in order to have more of the wheat berry included in the flour and to make a larger supply of flour available for human food. Some observations on the use of 80% extraction flour as a source of the B vitamins in the diet are included in this report.

EXPERIMENTAL PROCEDURE

The procedure described by Westerman and Hall ('47) was followed. Young albino rats weighing between 40 and 50 gm, of known hereditary and nutritional background, were placed on a B-complex free basal diet for 12 days to deplete their bodies of these vitamins. At the end of the depletion period the animals were divided into groups in such a manner as to have equal distribution in regard to sex and litter mates. Forty per cent of the sucrose in the basal diet was replaced by flour or ground whole wheat unless otherwise indicated. Four different experiments were conducted. The composition of all the diets is shown in table 1.

RESULTS AND DISCUSSION

First experiment: Enriched flour included in the diets at a 40% level with B vitamins added

In the first experiment diet I had choline and pyridoxine added to the enriched flour and diet II had choline, pyridoxine and riboflavin added (table 1). The analysis of the enriched flour showed that it contained 7.82 μg per gram of thiamine and 2.69 μg per gram of riboflavin. It supplied 18.77 μg of

TABLE 1

Composition of the diets

The B-complex free basal diet consisted of 20% vitamin free casein, 60% sucrose, 12% fat, 5% salt mixture and 3% cod liver oil. The flour replaced an equivalent amount of sucrose and was fed at a 40% level in the diet except where indicated. The B vitamins were added to the diet as indicated below.

DIET NO.	CHANGES IN DIET			
	Experiment I	Experiment II	Experiment III	Experiment IV
I	Enriched flour + 0.75 mg choline + 2 µg pyridoxine per gram	Whole wheat -	Whole wheat	Whole wheat
II	Enriched flour + 0.75 mg choline + 2 µg pyridoxine + 14.2 µg ribo- flavin per gram	Enriched flour	Whole wheat + 4.4 µg thiamine per gram	80% extraction flour
III		Enriched flour + 0.75 mg choline + 2 µg pyridoxine per gram	Enriched flour	80% extraction flour, enriched
IV	.	Enriched flour + 1 mg liver extract per gram	60% whole wheat	80% extraction flour, blended
V		Enriched flour + 0.75 mg choline + 2 µg pyridoxine + 14.2 µg ribo- flavin per gram	60% enriched flour	80% extraction flour, blended and enriched
VI		Enriched flour + 0.75 mg choline + 2 µg pyridoxine + 20.5 µg Ca pantothenate per gram	Enriched flour + 0.75 mg choline + 2 µg pyridoxine + 20.5 µg Ca pantothenate + 14.2 µg ribo- flavin per gram	Enriched flour
VII		Stock diet	Enriched flour + 0.75 mg choline + 2 µg pyridoxine + 20.5 µg Ca pantothenate + 14.2 µg ribo- flavin + 4.4 µg thiamine per gram	

thiamine and 6.46 μg of riboflavin per rat per day. Sure ('38) found that 10 μg of thiamine per day will meet the growth requirements of the rat. According to Edgar, Macrae and Vivanco ('37) the rat needs 40 μg per day of riboflavin for growth. Since the 6.46 μg of riboflavin furnished by the flour in diet II was not enough to support growth, it was decided to add 14.2 μg of riboflavin per gram to the enriched flour, thereby bringing the riboflavin intake per day up to 40.54 μg .

According to Tepley, Strong and Elvehjem ('42) flour contains approximately 3.5 μg per gram of pyridoxine. The flour in the diet provided a daily intake of 8.4 μg of pyridoxine. Lepkovsky ('38) reported that 10 μg of pyridoxine daily is necessary for optimal growth, so 2 μg of pyridoxine per gram was added to the flour. This would provide a total of 13.2 μg of pyridoxine per day, which should be ample for growth purposes.

The 0.75 mg of choline per gram was added to the flour to take care of any lack of this material in the diet. Griffith ('41) reported that with 18–24% of casein in the diet, hemorrhagic degeneration could be prevented by including 1–2 mg of choline in the daily ration. The diets used in these experiments allowed an intake of 1.7 mg choline per day. Dann ('41) reported that the rat synthesizes enough nicotinic acid for its needs, and Hundley ('47) found that tryptophane appeared to be a precursor of nicotinic acid. With casein in the diet to furnish tryptophane and the enriched flour providing nicotinic acid it was decided not to add this vitamin to the diet.

After the test was started the animals were weighed every 6 days over a 96-day period. The results are shown by the growth curves in figure 1. It will be noted that the animals on diet II, with the added riboflavin, made greater weight gains than those on diet I. The fur and general appearance of these animals were much better than those on diet I. While the addition of choline and pyridoxine to the flour may have had some effect in increasing the growth rate, the addition of a larger amount of riboflavin to the enriched flour along with

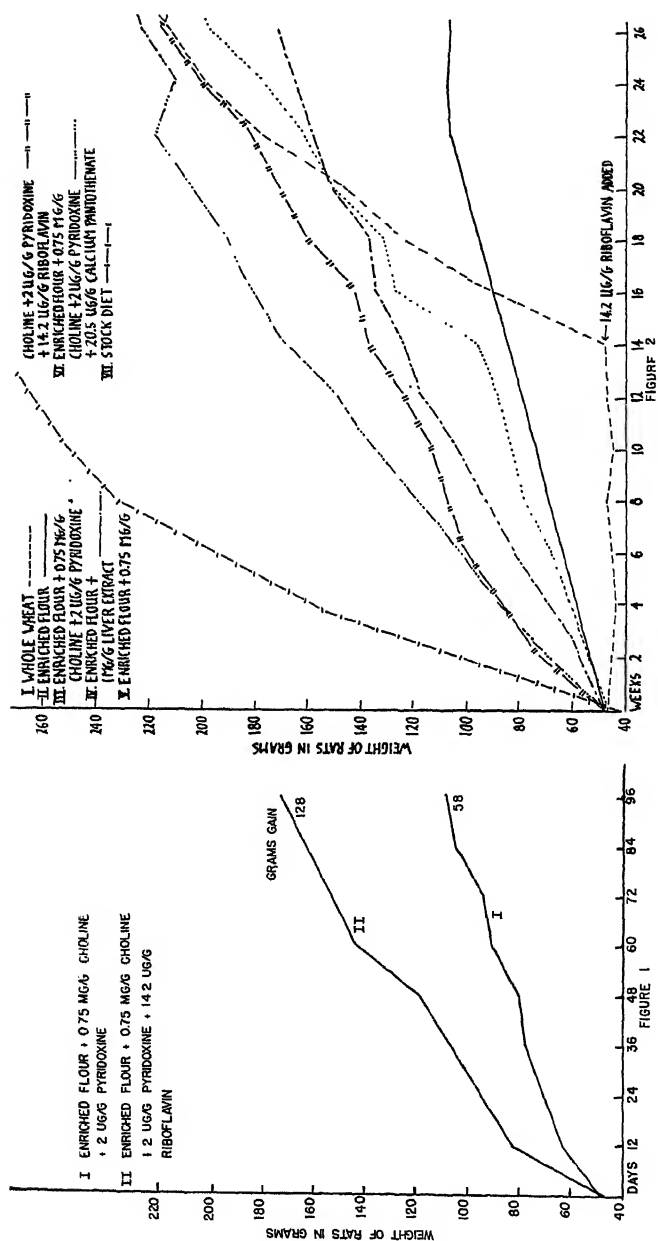


Fig. 1 Growth curves of animals in experiment I.

Fig. 2 Growth curves of animals in experiment II.

the choline and pyridoxine had a much more striking effect on the growth rate.

This experiment would seem to indicate that enriched flour did not contain enough riboflavin to support normal growth when included in the diet at a 40% level, as the addition of more riboflavin along with choline and pyridoxine aided in accelerating the growth rate.

Second experiment: Effect of the addition of B vitamins to enriched flour, on growth, maintenance and reproduction

The second experiment was set up on a long term basis to determine the growth rate, maintenance, reproduction and length of life of the animals fed the different diets. Seven diets (table 1) were used with 10 animals on each diet. Diets I and II, containing 40% whole wheat and enriched flour, respectively, and diet VII, the stock ration, were considered the control diets. Diet III had 0.75 mg choline and 2 μ g pyridoxine added per gram of enriched flour in order to meet the rat's requirements for these 2 materials. Diet IV had 1 mg of liver extract added per gram of enriched flour in order to furnish all the B vitamins. Diet V had 0.75 mg of choline, 2 μ g pyridoxine and 14.2 μ g riboflavin added to each gram of enriched flour in order to meet the requirement of the rat for these essentials. Diet VI was like diet III except that 20.5 μ g of calcium pantothenate was added per gram of enriched flour.

The average growth rates of the rats on the different diets are shown in figure 2. The animals surviving on diet I barely held their weight for 14 weeks of the test. They were denuded of fur, had a dermatitis on the feet and were inactive. These details are brought out on plate 1, A.

Analysis of the whole wheat showed that it contained 4.69 μ g of thiamine, 0.92 μ g riboflavin, 5.93 μ g of calcium pantothenate and 49.63 μ g of nicotinic acid per gram. The animals received 11.26 μ g of thiamine, 14.23 μ g of calcium pantothenate, 2.21 μ g of riboflavin and 119.11 μ g of nicotinic acid per day. The diet was low in calcium pantothenate since Supplee, Bender and

Kahlenberg ('42) reported that 20 μg of calcium pantothenate was needed for adequate growth. The riboflavin in the diet was very low, 2.21 μg per day. Inadequate calcium pantothenate and riboflavin undoubtedly accounted for the death of some of the animals on this diet and the loss of fur and failure to gain weight by the others.

At the beginning of the 15th week of the test, 14.2 μg per gram of riboflavin was added to the ground wheat used in diet I in order to bring the riboflavin intake nearer the normal amount required by the rat. All animals gained weight rapidly, as shown in figure 1. Ten weeks after the addition of riboflavin (24th week of the test), these animals had gained considerable weight. Their fur grew again; they looked and acted like normal animals (plate 1, B). They were not so large as those on the normal diet, which had made an average gain of 276 gm during the same period. The 2 females produced young but only 1 litter was raised.

At 3 months of age, 5 females of the second generation on diet I were bred but only 1 produced a litter. There were 10 young but these soon died. Evidently the diet containing whole wheat supplemented with riboflavin was sufficient for growth but not for reproduction and lactation in the second generation.

Diets II through VI contained enriched flour. The flour assayed 7.82 μg of thiamine, 2.69 μg riboflavin, 2.05 μg calcium pantothenate and 42.63 μg of nicotinic acid per gram. The diet provided 18.77 μg of thiamine, 6.46 μg riboflavin, 4.92 μg calcium pantothenate and 102.3 μg nicotinic acid daily.

The animals on diet II, enriched flour, made better weight gains than those on whole wheat. This may in part be explained by the increase in thiamine and riboflavin in this diet. However, the enriched flour furnished less calcium pantothenate and nicotinic acid than the whole wheat. The animals on diet II did not grow at a normal rate (fig. 2). By the end of 14 weeks they had developed a dermatitis on the paws, loss of fur, and curvature of the spine, as is shown on plate 1, C. They were bred but no young were born. As the test contin-

ued the dermal symptoms increased and some of the animals died. After 46 weeks 2 μ g per gram of pyridoxine was added to the enriched flour. This amount of pyridoxine added to that already in the flour was considered sufficient for growth and maintenance. Two weeks after this addition the fur began to grow, the dermatitis was cured, and weight was increased, but they failed to reproduce. This failure may be due to a lack of other vitamins.

Animals on diet III, with choline and pyridoxine added, averaged a slightly better growth rate than those on enriched flour alone (fig. 2). They lost part of their fur and showed the symptoms of a lack of calcium pantothenate, with reddish incrustations on the whiskers, head and paws. Three females had litters but destroyed their young soon after birth. This may have been due to a lack of riboflavin and calcium pantothenate.

Liver extract was added in diet IV in order to determine whether it would aid in promoting better growth than the addition of the synthetic vitamins. The animals on this diet made better weight gains than those on whole wheat or enriched flour (fig. 2). The growth was below normal; evidently not enough liver extract was added to provide the necessary essentials lacking in this diet.

The animals on diet V made better weight gains than those on diets I to IV (fig. 2; plate 2, A). However the animals on diet VI showed even greater weight gains (fig. 2). The amount of calcium pantothenate added plus the amount already in the flour brought the daily intake up to 54.12 μ g per rat per day in diet VI. This amount should more than take care of the growth requirement. Rats on this diet made the best growth of those on the experimental diets, but the average growth rate was not equal to that of the animals on the stock diet. These rats appeared active and their fur was in good condition (plate 2, B). The lack of riboflavin in this diet may account for the fact that these animals made less growth than those on the stock diet. The animals on diet VI were mated, but no young grew to maturity. This may have been due to

a lack of riboflavin in the diet, or not enough calcium pantothenate or other vitamins to allow for proper lactation.

Diet VII was the stock diet. Animals on this diet grew at a much faster rate than any of the other groups and showed no deficiency symptoms (fig. 2 and plate 2, C). They were able to produce normal litters and raise them to maturity, which would indicate that the diet was adequate.

This experiment was carried on over a 64-week period in order to compare the length of life of the rats on these diets. Seven of the 10 rats on the diet containing whole wheat were dead by the 14th week. Those on the enriched flour lived a longer time but most of them died in or before the 46th week. The addition of choline and pyridoxine in diet III did not seem to have much effect on the lengthening of life, since these animals died at about the same time as those on the enriched flour alone. The addition of choline, pyridoxine and riboflavin, as in diet V, and the addition of choline, pyridoxine and calcium pantothenate, as in diet VI, proved beneficial since 60 and 70%, respectively, of these animals lived for 64 weeks and then were discarded.

*Third experiment: Whole wheat and enriched flour at
the 40 and 60% levels*

In the third experiment it was decided to compare the effect on growth and reproduction of the animals on diets containing whole wheat, whole wheat with added thiamine, enriched flour, and enriched flour with the addition of sufficient B vitamins to meet the rat's requirements. The whole wheat and the enriched flour were included in the diets at the 40 and 60% levels. The 60% level was included since people in some parts of the world use cereals in the diet at this level. A series of animals on the stock diet were also included for the purpose of comparison. The composition of the diets is given in table 1 and the growth curves are shown in figure 3.

The animals with 40% enriched flour in the diet showed better weight gains than those on whole wheat. This is in accordance with an observation of Guerrant and Fardig ('47).

The addition of 4.4 μg of thiamine per gram to the whole wheat provided an approximate intake per rat per day of 22 μg of thiamine, which would be more than an ample supply of this vitamin for growth purposes. The animals with added thiamine in the diet did make better weight gains than those on whole wheat alone, thereby showing that whole wheat at a 40% level in the diet did not provide enough thiamine for normal growth. The animals with thiamine added to the whole wheat did not make quite such good gains throughout the test as those on enriched flour (fig. 3).

At a 60% level in the diet greater gains were made by the animals on enriched flour than by those on whole wheat (fig. 3). These gains were greater in each case than those of the animals with 40% of these constituents in the diet. This would seem to indicate that if the diets were lacking in B-complex vitamins a higher percentage of cereals in them would provide more of these substances.

It will be noted (fig. 3) that whole wheat at a 60% level in the diet promoted only slightly better growth than enriched flour at a 40% level. This may be due to the difference in vitamin content of the diet or a difference in digestibility of these 2 products. In regard to vitamin content, in diet IV with 60% whole wheat the animals received 16.78 μg of thiamine, 3.31 μg riboflavin, 21.41 μg of calcium pantothenate and 178.7 μg of nicotinic acid. Those on diet III with 40% enriched flour received 18.77 μg thiamine, 6.46 μg riboflavin, 4.92 μg calcium pantothenate and 102.3 μg nicotinic acid. The amount of thiamine obtained from diet IV is slightly less than that of diet III, while diet III contained about twice as much riboflavin, and diet IV had about 4 times as much calcium pantothenate as diet III. It may be possible that the increased calcium pantothenate in the whole wheat accounted for the added weight gain and in some way made up for the lack of riboflavin for growth purposes. The rats on diet V with 60% enriched flour obtained approximately 28.15 μg of thiamine, 9.68 μg riboflavin, 7.38 μg calcium pantothenate and 153.47 μg nicotinic acid per day. There was a higher content of thiamine

and riboflavin in diet V than in diet IV, but the calcium pantothenate content of diet IV was greater than that of V. The increased growth of the animals on diet V over that of the rats on diet IV may have been due to a better balance of the vitamins in the former diet. From our observations, when riboflavin is low and calcium pantothenate plentiful in the diet, along with the other vitamins, the growth is approximately that of animals with lowered amounts of calcium pantothenate and a plentiful supply of riboflavin; but even better growth is obtained when these vitamins are in the diet.

The rats on diets I through V were mated but no young were produced. It may be that the diets were lacking in vitamin E, but it would seem that whole wheat would have enough vitamin E in the germ portion to promote reproduction. It is possible that the lack of some of the B-complex vitamins may have been the cause.

At the beginning of the 20th week of the test the animals on diets III, IV and V were each divided into 2 groups. One group had 4% autoclaved yeast added to the diet and the other had 10% autoclaved peanuts added. These products replaced an equivalent amount of sucrose in the diet. It will be noted (fig. 3) that the autoclaved peanuts did not contain materials needed by the rat, as growth was decreased or remained at the same rate. In the case of the addition of autoclaved yeast, growth was accelerated in all cases, which indicated that the yeast contained substances besides thiamine which had a beneficial effect upon growth.

Diet VI had choline, riboflavin, pyridoxine and calcium pantothenate added to the enriched flour while diet VII had these same additions plus 4.4 μ g of thiamine per gram. The growth curves indicate that the animals on these 2 diets approached the same rate of growth as those on the stock diet. At the end of the 20th week the average weights were 235, 267, and 281 gm, respectively. Since the animals on diet VII made better gains than those on diet VI, it seems evident that the addition of the thiamine was beneficial. None of the rats

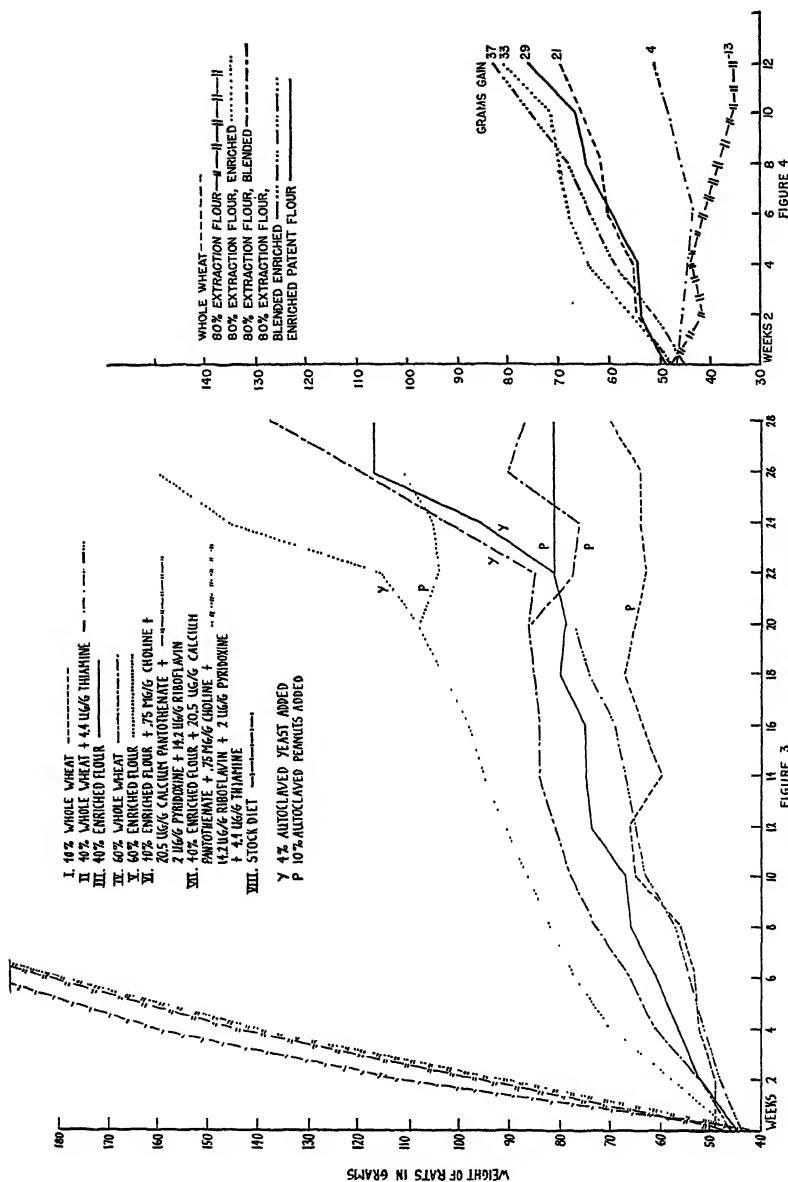


Fig. 3 Growth curves of animals in experiment III.

Fig. 4 Growth curves of animals in experiment IV.

on the 2 diets made quite so much gain throughout the test as those on the stock diet.

At the beginning of the 14th week of the test, the animals on these diets were mated. Three of the 4 females on diet VI had litters but destroyed the young. This happened again in the second mating which would seem to indicate that the diet was not adequate for raising the young.

Of the rats on diet VII, with thiamine added, 3 out of 4 females produced litters. The young were weaned at the age of 21 days and kept on diet VII for a growth test. Their average growth rate approximated that of the first generation. By the 14th week after weaning, they had an average weight of 216 gm while those of the first generation at the same age weighed 221 gm. The animals were mated at this time and given 3 mg of vitamin E each day to make up for any deficiency of this vitamin in these diets. None of the second generation animals produced live young. The diet was evidently lacking in some other factor necessary for reproduction and lactation.

*Fourth experiment: Effect on growth of 80%
extraction flour*

When the government began the policy of shipping more wheat and flour abroad it became necessary to conserve these materials at home. In order to do this the government made it mandatory that 80% extraction flour be milled in this country for a time. Immediately the question was raised as to whether or not this type of flour would have more nutritive value. An experiment was set up to compare the effect on the growth of the rat when fed a diet containing whole wheat, enriched patent flour, and 80% extraction flour. The whole wheat was taken from the same sample as that used in milling the 80% extraction flour. The enriched patent flour was a part of the same used in the previous experiments, obtained from a commercial mill.

The 80% extraction flour was milled by the Department of Milling Industry of the college. Since this was a change in

the processing, it was suggested that it might be difficult to set the mill to obtain exactly 80% extraction flour; therefore included in the test was an 80% extraction flour which was blended from flour milled at 5 different mills. Also it was decided to include 80% extraction flour which had been enriched to the same level with the B-complex vitamins as the enriched patent flour. It was thought this might show whether or not there were enough of the other B-complex vitamins in the 80% extraction flour to promote as good growth as the enriched patent flour.

To determine the amount of each vitamin needed to enrich the 80% extraction flour, analyses were made of the enriched patent flour and of the 80% extraction flour. The enriched patent flour contained 7.82 μg of thiamine, 3.32 μg of riboflavin and 44.2 μg of nicotinic acid per gram. The 80% extraction flour contained 3.28 μg of thiamine, 0.32 μg of riboflavin, and 10.0 μg of nicotinic acid per gram. Therefore it was necessary to add 4.54 μg of thiamine, 3.00 μg of riboflavin, and 34.2 μg of nicotinic acid per gram to the 80% extraction flour in order to bring the content of these vitamins up to the same levels.

The 6 different diets used in the experiment contained the whole wheat and flour at a 40% level. The entire composition of the diets is given in table 1. The growth tests were conducted over a period of 12 weeks and the results are shown in figure 4. The animals on diet II with the 80% extraction flour without enrichment made no weight gains; instead, they lost 13 gm in weight (fig. 4). Animals of diet IV with 80% extraction flour, blended, also showed very poor growth and gained only 4 gm during the test. The animals on both of these diets showed the B-complex deficiency symptoms of inactivity, loss of appetite, nervousness and in some cases paralysis.

Diet I, containing whole wheat, produced an average weight gain of 21 gm during the test period while the animals on diet VI with enriched flour gained an average of 29 gm. This is in accord with the previous tests where the animals on enriched flour at a 40% level in the diet made better weight gains than those on whole wheat. The rats on diets III and V with the

enriching agents added to the 80% extraction flour showed slightly better weight gains throughout the test than those with enriched flour in the diet; their average gains were 33 and 37 gm, respectively.

The results would seem to indicate that if 80% extraction flour is to be used as a source of B-complex vitamins in the diet, it should be enriched; then it is only slightly better in promoting growth in rats than is the enriched patent flour.

SUMMARY

Experiments have been conducted on albino rats to determine the effect on growth, maintenance, reproduction and length of life of the use of whole wheat, enriched flour, enriched flour supplemented with other B-complex vitamins, and 80% extraction flour in the diets. Under the conditions of the experiments it was found that the whole wheat and the enriched flour did not support normal growth and reproduction in the rats when included in the diet as sources of the B-complex vitamins, at either the 40 or 60% level. However, the enriched flour in the diet at these levels promoted better growth than the whole wheat.

Under the conditions of these experiments the enriched flour supplemented further with the B vitamins supported better growth, maintenance, reproduction and length of life than the enriched flour alone. The addition of choline, pyridoxine and riboflavin to the enriched flour resulted in greater weight gains than the addition of choline and pyridoxine. However, when choline, pyridoxine and calcium pantothenate were added even better growth was obtained. The animals with additional riboflavin or calcium pantothenate in the diet lived longer and showed fewer deficiency symptoms than those on the diet of enriched flour with only choline and pyridoxine added.

Animals on diets with choline, pyridoxine, calcium pantothenate and riboflavin added to the enriched flour, and these vitamins plus thiamine, made excellent growth records which compared favorably with the average gain made by the ani-

mals on the stock diet. None of the animals on the test diets reproduced so well as those on the stock diets.

The 80% extraction flour, without enrichment, did not support growth in the rats. When 80% extraction flour was enriched at the same levels as patent flour it supported slightly better growth than the enriched patent flour.

ACKNOWLEDGMENTS

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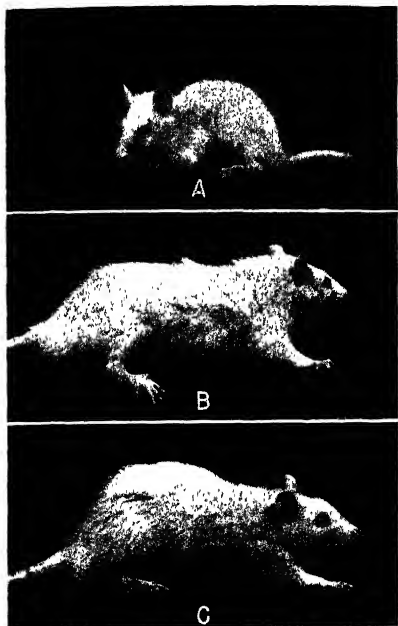


PLATE 1

EXPLANATION OF FIGURES

- A. After 14 weeks on diet I containing 40% whole wheat.
B. Improvement shown 10 weeks after addition of riboflavin to diet I.
C. After 24 weeks on diet II containing 40% enriched flour.

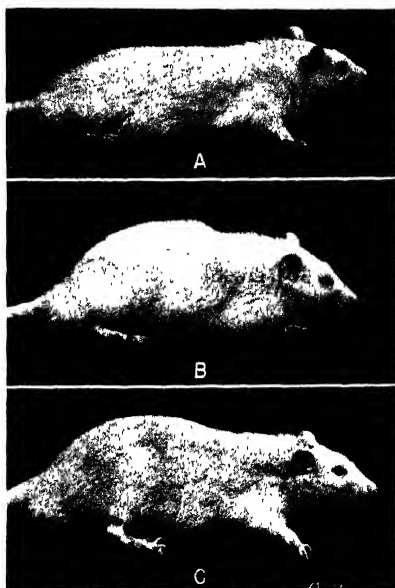


PLATE 2

EXPLANATION OF FIGURES

- A. After 24 weeks on diet V containing 40% enriched flour with choline, pyridoxine and riboflavin added.
B. After 24 weeks on diet VI containing 40% enriched flour with choline, pyridoxine and calcium pantothenate added.
C. After 24 weeks on the stock diet.

THE RELATION OF ASCORBIC ACID METABOLISM IN THE RAT TO DIETS HIGH IN PROTEIN, CARBOHYDRATE OR FAT¹

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Ascorbic acid deficiency has long been associated with disturbances in protein metabolism, particularly the formation of intercellular cementing substance. Sealock and Silberstein ('39) were the first workers to connect this deficiency with the metabolism of specific amino acids. The normal metabolism of certain amino acids is disturbed in scorbutic guinea pigs and immature infants (Levine, Gordon and Marples, '41; Christensen and Lynch, '48). Administration of ascorbic acid, often in large amounts, restores normal metabolism. In the present experiments we have attempted to determine whether ascorbic acid metabolism can be influenced in the rat by increasing protein metabolism while maintaining caloric intake constant.

Rats, therefore, were fed diets high in carbohydrate, fat or protein but in all cases adequate to maintain nitrogen balance, and the ascorbic acid concentrations in various organs determined.

METHODS

Adult male rats of the Sprague-Dawley strain were fed for 5 weeks by the stomach tube technique of Reinecke, Ball and Samuels ('39). The composition of the diets used is given in table 1.

¹ This investigation was supported by grants from the Sugar Research Foundation and from the Medical Research Fund, University of Utah.

In addition, in experiment 2 the protein-fed rats were given 6 mg ascorbic acid per day. In experiment 3 the fat group and 1 protein group received 50 mg ascorbic acid per day by mouth.

At the end of the feeding period the rats were anesthetized with sodium amytal given intraperitoneally, 9 mg/100 gm

TABLE 1
Diets used in stomach tube feeding

	HIGH FAT	HIGH CARBOHYDRATE	HIGH PROTEIN
Lactalbumin, Labco (gm)	30	30	263.25
Gelatin, U.S.P. (gm)	30	30	30
Corn oil (gm)	105.8	3	3
Dextrin (gm)	0	233.25	0
Salt mixture, U.S.P. no. 1 (gm)	12.0	12.0	12.0
Oleum Percomorphum, reinforced (drops) ¹	12	12	12
Vitamin concentrate (gm) ²	6.0	6.0	6.0
Water to make (ml)	900	900	900
Calories (per ml)	1.30	1.31	1.30
Caloric distribution (% of total calories)			
Protein	19.5	19.5	92.4
Fat	80	2.7	2.7
Carbohydrate	0.5	77.8	4.9

¹ Mead Johnson and Co.

² Lederle's Lederplex. According to label, 6 gm contained (in milligrams) thiamine hydrochloride 24, riboflavin 24, nicotinamide 120, pyridoxine 2.4, calcium pantothenate 36, choline 240, inositol 120 and folic acid 2.4; plus other water-soluble extractives from 96 gm liver.

body weight. The abdomen was then opened and blood withdrawn from the abdominal aorta. The various organs were removed, weighed, ground with sand and 6% trichloroacetic acid and centrifuged. The supernatant solutions and blood plasma were analyzed for ascorbic acid by the method of Roe and Kuether ('43).

A sample of liver was also analyzed for total nitrogen by the Kjeldahl method.

RESULTS

As can be seen in table 2, there were no significant differences in the ascorbic acid concentrations of any of the tissues when the fat-fed and carbohydrate-fed groups were compared, with the possible exception of the livers in experiment 1. In this case, when ascorbic acid was related to the nitrogen content of the livers, this difference was eliminated. Apparently a wide shift in the ratio of carbohydrate to fat as sources of calories did not affect ascorbic acid metabolism in a marked way.

On the other hand, the protein-fed groups showed significant differences from those on the other 2 diets. The ascorbic acid concentrations of muscle and blood were always lower in the animals receiving the high protein diet than in the other groups. The administration of high doses of ascorbic acid did not alter blood levels significantly, but the concentration in the muscles was definitely increased.

The kidneys and livers of protein-fed rats also had lower concentrations of ascorbic acid on the unsupplemented diets. Both organs, however, were larger in the animals on the high protein diets. When considered on the basis of ascorbic acid per 100 gm body weight, there was as much in the livers of the protein-fed rats as in the carbohydrate- or fat-fed groups; the lower concentration in the liver was offset by the larger size of the organ. This was not true in the case of the kidney; the concentration per 100 gm body weight was also significantly lower unless ascorbic acid was administered. The levels in both kidneys and liver were raised when the vitamin was fed.

As shown in table 3, the significant differences in total weight of organs are those which have been previously reported (Osborne, Mendel, Park and Winternitz, '25). The livers of the rats fed high protein were largest in experiment 2 where the caloric intake was also largest. In experiment 1, the livers of the fat-fed and protein-fed rats were larger than those of the rats fed carbohydrate but did not differ

Ascorbic acid content of organs of male rats on high fat, high carbohydrate or high protein diets¹

EXPERIMENT AND DIET	NO. RATS	LIVER		KIDNEY		BRAIN		ADRENALS		BLOOD		MUSCLE
		mg per gm Organ	mg per gm N 100 gm BW ²	mg per gm Organ	mg per 100 gm BW ²	mg per gm Organ	mg per 100 gm BW ²	mg per gm Organ	mg per 100 gm BW ²	mg per 100 ml	mg per gm Tissue	
<i>Experiment 1</i>												
Fat diet	10	0.242 ± .017	7.84 ± 0.71	0.153 ± .006	0.102 ± .005	0.252 ± .010	0.145 ± .005	3.19 ± .22	0.054 ± .0053	0.90 ± .04	0.037 ± .0019	
Carbohydrate diet	10	0.262 ± .014	7.47 ± 0.53	0.130 ± .010	0.084 ± .005	0.228 ± .019	0.137 ± .011	3.21 ± .14	0.052 ± .0037	0.94 ± .06	0.035 ± .0013	
Protein diet	8	0.188 ± .010	5.27 ± 0.20	0.068 ± .004	0.064 ± .004	0.227 ± .013	0.133 ± .008	3.19 ± .18	0.069 ± .0030	0.79 ± .05	0.026 ± .0010	
<i>Experiment 2</i>												
Fat diet	5	0.289 ± .001		0.137 ± .005	0.091 ± .004	0.368 ± .018	0.220 ± .011	3.86 ± .11	0.089 ± .0048	0.83 ± .06	0.025 ± .0003	
Carbohydrate diet	7	0.337 ± .013		0.143 ± .006	0.092 ± .004	0.380 ± .012	0.232 ± .011	3.31 ± .20	0.064 ± .0055	0.83 ± .06	0.025 ± .0008	
Protein diet	5	0.222 ± .011		0.088 ± .004	0.085 ± .005	0.334 ± .008	0.172 ± .008	3.89 ± .13	0.079 ± .0060	0.65 ± .07	0.019 ± .0006	
Protein diet + 6 mg ascorbic acid injected	6	0.237 ± .024		0.092 ± .007	0.092 ± .004	0.326 ± .022	0.183 ± .010	4.06 ± .13	0.083 ± .0033	0.64 ± .05	0.023 ± .0018	
<i>Experiment 3</i>												
Fat diet + 50 mg ascorbic acid fed	7	0.331 ± .008		0.221 ± .006	0.134 ± .006	0.323 ± .007	0.195 ± .008	3.79 ± .18	0.072 ± .0031	0.96 ± .04	0.031 ± .0018	
Protein diet	7	0.278 ± .016		0.131 ± .003	0.101 ± .002	0.308 ± .014	0.180 ± .011	3.57 ± .18	0.062 ± .0046	0.76 ± .06	0.024 ± .0010	
Protein diet + 50 mg ascorbic acid fed	8	0.303 ± .015		0.157 ± .007	0.124 ± .005	0.310 ± .006	0.180 ± .005	3.86 ± .16	0.064 ± .0026	0.80 ± .04	0.028 ± .0007	

¹ All values are given as means with the standard errors of the means.

² Total body weight of animal at autopsy.

TABLE 3
Weights of organs of rats on high fat, high carbohydrate, or high protein diets fed by stomach tube

DIET	RATS	CALORIES PER DAY	AVE. WT. AT START	CAL./100 GM/DAY	AVE. WT. AT END	LIVER ¹ gm	% N	KIDNEYS ¹ gm	ADRENALS gm	BRAIN gm	THY- MUS gm
<i>Experiment 1</i>											
Fat diet	10	50.1	252	19.9	299	10.62 ± .46	3.30 ± .05	2.016 ± .081	0.050	1.726	0.214
Carbohydrate diet											
	10	48.0	249	19.3	285	7.91 ± .26	3.58 ± .05	1.878 ± .065	0.044	1.712	0.285
Protein diet	8	48.0	247	19.4	282	10.07 ± .30	3.72 ± .06	2.636 ± .101	0.050	1.876	0.211
<i>Experiment 2</i>											
Fat diet	5	48.0	296	16.2	291	8.97 ± .32	3.28 ± .20	1.944 ± .034	0.067	1.748	
Carbohydrate diet											
	7	48.0	295	16.3	292	7.61 ± .18	3.55 ± .03	1.884 ± .193	0.055	1.801	
Protein diet	5	55.0	304	18.1	338	12.14 ± .62	3.59 ± .03	3.269 ± .139	0.069	1.731	
Prot. and 6 mg ascorbic acid injected											
	6	52.0	286	18.2	318	12.28 ± .42	3.54 ± .06	3.183 ± .144	0.062	1.771	
<i>Experiment 3</i>											
Fat + 50 mg ascorbic acid fed	7	48.0	297	16.2	289	7.66 ± .29	3.59 ± .07	1.740 ± .053	0.056	1.732	
Protein diet	7	48.0	298	16.1	293	8.37 ± .37	3.84 ± .02	2.270 ± .077	0.050	1.700	
Protein + 50 mg ascorbic acid fed	8	48.0	308	15.6	296	7.97 ± .18	3.80 ± .04	2.364 ± .037	0.050	1.752	

¹ Values are given as means with the standard errors of the means.

significantly between themselves. However, the nitrogen content of the livers of the protein-fed groups was always higher than that in the other groups of all 3 experiments. In experiment 1 where all rats received equicaloric rations in quantities sufficient to cause gains in weight, the rats receiving fat had accumulated enough lipid in the liver to offset the increased protein content of the livers of the protein-fed rats. In experiment 3 where the animals were slightly underfed, the livers tended to be smaller and to have higher nitrogen contents, but the difference between diets was still present.

The kidneys of the protein-fed rats were always larger. The difference was more marked if the calories on the high protein diet were sufficient for gains in weight, but it was still marked in experiment 3 where food intake was insufficient to maintain weight.

There was no significant difference in the size of the adrenals of the protein- and fat-fed rats. The average weight of the adrenals of the carbohydrate-fed animals was slightly smaller. These results confirm the observations of Ingle, Ginter and Nezamis ('43) that, in the adult rat, there is no significant increase in adrenal size on a protein diet. Since in these experiments 92% of the calories was obtained from protein it seems unlikely that the adrenals play any role in the catabolism of dietary protein; otherwise, some evidence of hypertrophy would have been expected.

DISCUSSION

There seems to be some effect of protein diet on the metabolism of ascorbic acid in the rat. While there is no definite evidence of the mechanism involved, it seems that the following hypothesis would most simply fit the facts:

As Sealock, Perkinson and Silberstein ('40) have shown, ascorbic acid is related to the metabolism of tyrosine and phenylalanine. It also appears to be involved in the metabolism of glutamine and glycine (Christensen and Lynch, '48). On the high protein diet used here relatively large amounts of these amino acids, as well as all others, were catabolized.

In such a situation it might be expected that the utilization of ascorbic acid would be greatly increased. Production, in which the liver appears to play a prominent role (Smythe and King, '42), could not keep up with destruction, and concentrations in the plasma and in the tissues which would metabolize such amino acids decreased. In those tissues like the brain, which did not use these amino acids, or in those tissues where ascorbic acid appears to be associated with some other specific metabolic process, such as the adrenals, there was no significant effect of the diet.

When ascorbic acid was fed, the concentrations in the tissues where they were low were increased, but the vitamin was still removed so rapidly by these tissues that the plasma level did not rise significantly. This would be expected, since the distribution of ascorbic acid to the tissues is not simply a diffusion phenomenon, but must involve considerable energy utilization to maintain the marked concentration gradient. If there was an increase in the processes utilizing ascorbic acid it might be expected that there would be energy available for maintaining an increased concentration gradient. In no single tissue did this gradient reach the maximum for that tissue found by Kuether, Telford and Roe ('44) in guinea pigs.

On the other hand, if decreased production were primarily responsible for the low ascorbic acid values, one would have expected that the levels in all tissues, whether they utilized amino acids or not, would be decreased. This is seen in the scorbutic guinea pig where the levels in the brain and adrenals follow the general drop in tissue concentration. One would also have expected the circulating fluids to show the effect of dietary ascorbic acid, if tissue demand were normal and liver production alone decreased. Also, as Roberts and Spiegel '47) demonstrated, the production of ascorbic acid, like other functions of the liver, is dependent on the supply of sulphur-containing amino acids. These were not lacking in any of the diets, and certainly not in the high protein ration. A relationship between tyrosine catabolism and ascorbic acid has been

demonstrated in liver and kidney tissues by Lan and Sealock ('44). It probably is also true for muscle since the aromatic amino acids undergo metabolism there. It seems most logical, therefore, to assume that the changes observed were due to increased destruction of ascorbic acid in the presence of high amino acid metabolism.

Heinemann ('36) studied the influence of a diet containing 50% of the calories as protein on the excretion of, and requirement for, ascorbic acid in the human being. A diet containing 11% of the calories as protein was used as the basis of comparison. He found no effect. Since our high protein diets contained 92% of the calories as protein the apparent difference in results may be due to the greater sustained protein level in our experiments. It may also be due to the different methods of study or to difference in species. Certainly there seems to be an influence of high protein diets on the tissue distribution of ascorbic acid in the rat.

CONCLUSIONS

A high protein diet led to a decrease in the concentration of ascorbic acid in tissues which metabolize considerable amounts of amino acids, such as the liver, kidneys and muscle. The levels in the plasma were also decreased.

There was no change in brain or adrenal ascorbic acid concentrations.

The enlargement of the liver on a high protein diet was sufficient to leave no significant difference in the amounts of liver ascorbic acid per unit body weight. This was not true of the kidney. This organ showed a marked increase in size on the high protein diet, but the concentrations of ascorbic acid were so low that there was less ascorbate per unit of body weight.

Administration of ascorbic acid led to an increase in the concentration of ascorbate in the liver, kidneys and muscle of protein-fed rats, but even doses of 50 mg a day did not significantly increase the concentration in the plasma.

It is thought that the results are best explained by an increased utilization of ascorbic acid in those tissues able to

metabolize large amounts of amino acids. The muscle illustrates the changes in a tissue metabolizing both ascorbate and protein, the blood reflects the body balance, and the liver shows the compensation changes of a producing organ.

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THE NEED FOR AND INTERRELATIONSHIP OF FOLIC ACID, ANTI-PERNICIOUS ANEMIA LIVER EXTRACT, AND BIOTIN IN THE PIG

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ONE FIGURE

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Available literature on attempted experimental production of anemia in animals was reviewed in an article by Miller and Rhoads ('35). These workers found that feeding a modified canine-black-tongue-producing diet to swine produced a symptom-complex marked by oral mucus membrane lesions, achlorhydria, and anemia. There was remission of the anemia and amelioration of symptoms as a result of oral or parenteral administration of liver extract.

Cartwright, Wintrobe and Humphreys ('46) using a purified diet containing 2% sulfasuxidine in the ration, produced in a pig an anemia which responded to a highly purified liver extract but not to biotin. They suggested that in the pig a "folic acid" deficiency may be associated with the develop-

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ment of a normocytic anemia. In the rat, a similar dietary regimen produced a deficiency in growth that responded promptly to a combination of biotin and pteroylglutamic acid and showed partial response to a 1% level of either yeast concentrate or liver extract (Welch and Wright, '43).

Welch et al. ('47) by use of a crude chemical antagonist that interfered with the metabolism of pteroylglutamic acid in the pig, interrupted and significantly inhibited the formation of erythrocytes and granulocytes. The addition of a crude source of extrinsic factor, together with normal human gastric juice, was found to allèviate the interference.

Cunha et al. ('47) failed to produce an anemia in swine by use of a purified diet including sulfathalidine for a 5-week period at a 0.5% level supplemented with 6 B-complex vitamins. They found that the addition of "folic acid" or p-aminobenzoic acid (PABA) alone to the basal purified ration stimulated hemoglobin formation to a small extent. However, when folic acid was added to the purified basal ration in addition to an anti-pernicious anemia liver extract or other vitamins (inositol, biotin, PABA), it did not stimulate hemoglobin formation, possibly indicating some vitamin imbalance.

The experiment reported herein gives data on the production of a folic acid deficiency with the pig and on an interrelationship of pteroylglutamic acid and an anti-pernicious anemia liver extract with biotin. In addition, folic acid and the anti-pernicious anemia liver extract are compared in preventing an anemia which developed in the pig.

EXPERIMENTAL

Six-week-old purebred Chester White pigs were used in this experiment. They were divided equally in each lot as to condition, weight, sex, and age.

The rations fed are shown in tables 1 and 2. The same pigs were used throughout the 2 phases of this experiment. During the first phase (13 weeks) the effect of sulfasuxidine on the need for folic acid by the pig was studied. Also, the effect of

using a liver preparation,⁴ and the addition of folic acid alone and in combination with biotin and 10% of dehydrated alfalfa meal to the basal ration was investigated. When alfalfa meal was added to the basal ration the amount of casein was lowered so that the total protein content of the ration was the same

TABLE 1

The effect of sulfasuxidine (S) on the folic acid (FA), anti-pernicious anemia liver extract (APLE), and biotin (B) needs of the pig

RATION FED	BASAL ONLY ¹	BASAL + S ²	BASAL + S + FA ³	BASAL + S + APLE ⁴	BASAL + S + FA + B ⁵	BASAL + 10% ALFALFA
Lot number	I	II	III	IV	V	VI
Number of pigs ^a	3	3	4	4	4	4
Av. daily gain (lbs.)	0.72	0.61	0.65	0.67	0.71	0.70
Av. daily feed consumption (lbs.)	1.34	1.34	1.35	1.37	1.36	1.36
Lbs. of feed per lb. of gain	1.87	2.20	2.10	2.04	1.91	1.96

¹ Basal ration contained 6 B-complex vitamins (thiamine, riboflavin, niacin, pyridoxine, pantothenic acid and choline).

² Sulfasuxidine fed at a level of 2% of the ration.

³ Crystalline folic acid was fed in both lots at a level of 50 µg per 100 gm of feed.

⁴ Injected intramuscularly, in the gluteal muscle, every second day. It was injected at the rate of 0.1 ml daily (1 U.S.P. injectable unit).

⁵ Biotin was fed at a level of 20 µg per 100 gm of feed.

^a These pigs were 6 weeks old and averaged approximately 24 lbs. in weight at initiation of this 13-week trial.

as the basal ration. During the second phase (8 weeks) the effect of a crude folic acid antagonist⁵ preparation on the need for folic acid by the pig was studied.

The purified basal ration used in this experiment was the same as that reported previously by Heinemann, Ensminger,

⁴ "Refined Solution Liver Extract, Parenteral" used for treatment of human pernicious anemia. Made from pork. Supplied by Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y.

⁵ The crude folic acid antagonist (batch N67) was obtained from Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y.

TABLE 2

The effect of sulfasuxidine (S) and crude folic acid antagonist (ANT) on the folic acid (FA), anti-pernicious anemia liver extract (APLE), and biotin (B) needs of the pig

RATION FED	BASAL ONLY ¹	BASAL + S ²	BASAL + S + ANT ³	BASAL + S + FA ⁴	BASAL + S + ANT + FA ⁵	BASAL + S + APLE ⁶	BASAL + S + APLE ⁷ + APLE ⁷	BASAL + S + FA + B ⁸	BASAL + S + ANT + FA + B
Lot number	I	IIA	IIB	IIIA	IIIB	IVA	IVB	VA	VB
Number of pigs ⁹	3	1	2	2	2	2	1	2	2
Av. initial wt. (lbs.)	88.3	89	73	94	73	84	93	95	83
Av. daily gain (lbs.)	0.66	0.41	0.44	0.39	-0.19	0.23	0.04	0.63	0.49
Av. daily feed consumption (lbs.)	2.04	2.01	1.78	1.96	1.27	1.86	1.39	2.06	1.77
Lbs. of feed per lb. gain	3.09	4.89	4.07	4.99	.	8.00	38.93	3.25	3.60

¹ Basal ration contained 6 B-complex vitamins (thiamine, riboflavin, niacin, pyridoxine, pantothenic acid and choline).
² Sulfasuxidine was fed at a level of 2% of the ration.

³ Crude folic acid antagonist was fed at a level of 0.2% of the ration.

⁴ Crystalline folic acid (no antagonist fed) was given at a level of 50 µg per 100 gm of feed.

⁵ Crystalline folic acid (when antagonist was fed) was given at a level of 100 µg per 100 gm of feed.

⁶ Injected intramuscularly, in the gluteal muscle, every second day. It was injected at the rate of 0.1 ml daily (no antagonist fed) (1 U.S.P. injectable unit).

⁷ Injected intramuscularly, in the gluteal muscle, every second day. It was injected at the rate of 0.2 ml daily (when antagonist was fed) (2 U.S.P. injectable units).

⁸ Biotin was fed at a level of 20 µg per 100 gm of feed.

⁹ These pigs were 19 weeks old at the initiation of this 8-week trial.

Cunha and McCulloch ('46). The ration consisted of casein⁶ 26.1%, sucrose 57.7%, lard 11%, and mineral mix⁷ 5.2%. Water soluble vitamins were fed (in mg per kg of live weight per day) as follows: thiamine 0.52, riboflavin 0.12, niacin 1.20, pantothenic acid 0.50, pyridoxine 0.20, and choline chloride 10.00; fat soluble vitamins were supplied (per pig daily) as follows: vitamin A, 5,000 I.U., vitamin E, 57 mg, vitamin K, 2 mg, vitamin C, 50 mg, and vitamin D, 700 I.U.

A modification of the "paired feeding technique" (Mitchell and Beadles, '30) was used in order to maintain the same feed intake by all pigs fed purified rations. Thus, the feed intake of the pigs was limited to the amount consumed by the pig with the least appetite — with the vitamins and the sulfonamide studied being the only variables among the lots of pigs. Later in the experiment as the appetite varied so much between lots of pigs, the paired feeding technique was altered. The pigs were fed in individual feeding stalls. To prevent coprophagy the pigs were kept on raised floors which were washed twice daily. At no time during the experiment was any evidence of coprophagy observed.

To prevent rancidity and subsequent destruction of vitamins, the purified ration was mixed every third day and kept in an ice box. All vitamin solutions were stored under refrigeration. The required amount of the vitamins fed each pig was measured in calibrated pipettes and placed on top of the ration every other day (every fourth feeding) just before feeding time.

Each pig was bled weekly from the marginal ear vein. The blood examined was for hemoglobin, erythrocyte, leucocyte, and differential cell counts as well as reticulocytes.

Pipettes were filled from the flowing blood on the ear. Smears were prepared from the same source, the stagnant blood being wiped away as it gathered. Hemoglobin was determined according to the method of Evelyn ('36). Leuco-

⁶ Vitamin Test Casein GBI, manufactured by General Biochemicals, Inc., Chagrin Falls, Ohio. Alcohol extracted.

⁷ Same as used by Wintrobe ('39), and as modified by Heinemann et al. ('46).

cytes were counted in 2% acetic acid diluent tinted with gentian violet. Erythrocytes were diluted with Hayem's solution. Smears from differential leucocyte counts were stained with Wright's stain and 200 cells were examined. Reticulocyte smears were stained with brilliant cresyl blue and counter-stained with Wright's stain.

RESULTS AND DISCUSSION

The data in table 1, during the first 13-week trial, show that no beneficial effect on growth or efficiency of feed utilization was obtained when the anti-pernicious anemia liver extract, or folic acid alone and in combination with biotin, was added to a purified ration containing sulfasuxidine. The addition of 10% dehydrated alfalfa meal to the purified basal ration resulted in no beneficial effect on growth or efficiency of feed utilization.

In lots III and IV where folic acid and the anti-pernicious anemia liver extract were fed, biotin deficiency symptoms showed up 2 weeks earlier (after 5 weeks on experiment) than in lot II where the pigs were fed the basal ration plus sulfasuxidine. The biotin deficiency symptoms obtained were similar to those described by Lindley and Cunha ('46) and Cunha et al. ('46). The pigs in lots III and IV developed much more severe biotin deficiency symptoms than the pigs in lot II. Apparently, the addition of either folic acid or the anti-pernicious anemia liver extract to the basal ration plus sulfasuxidine caused biotin deficiency symptoms to appear sooner and to develop much more severely (fig. 1). This may have been caused by some vitamin imbalance. Suggestions of a vitamin imbalance were obtained by Cunha et al. with rats ('43), with sows ('44), and young pigs ('47), and by Richards with rats ('45). No biotin deficiency symptoms were obtained in lots I, V, and VI.

In both phases of this experiment, occasional scouring, characterized by grayish feces, was obtained in all lots of pigs fed the purified ration except for the pigs fed the basal ration + 10% alfalfa which was fed only during the first phase. The

scouring usually lasted from 36 to 48 hours and did not seem to have any effect on appetite. Similar scours were prevented last year by including sulfathalidine in the ration at a 0.5% level (Cunha et al., '47). However, sulfasuxidine at the level fed in this experiment did not prevent the scouring which occasionally occurs with the basal ration used.

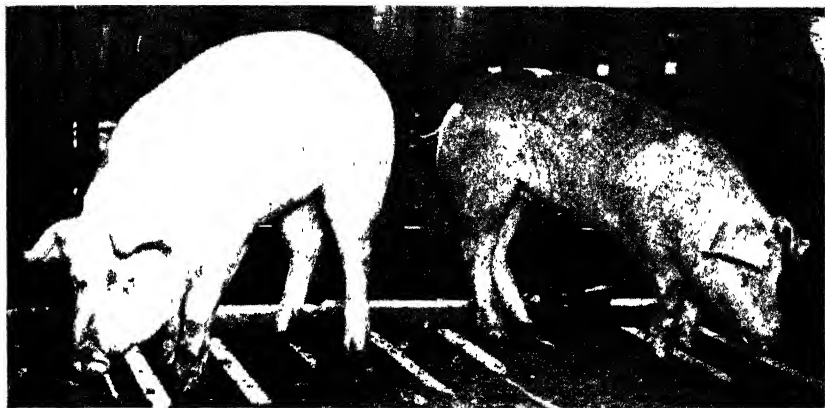


Fig. 1 During the first 13 weeks on experiment, the pig on the right was fed the basal ration + sulfasuxidine + folic acid. The pig on the left was fed the same ration but without folic acid. This picture illustrates the effect of folic acid in increasing the pig's need for biotin as shown by the pig on the right exhibiting severe biotin deficiency symptoms. Similar results in increasing biotin needs for the pig were obtained when an anti-pernicious anemia liver extract was added to the basal ration + sulfasuxidine.

The data given in table 2 show the results obtained during the second phase of the experiment which lasted for 8 weeks. The detrimental effect of feeding sulfasuxidine over a long period of time is evidenced by the decreased growth rate and increased feed required per pound of gain. The addition of a crude folic acid antagonist preparation to the basal ration plus sulfasuxidine did not lower gains or efficiency of feed utilization as compared with the feeding of sulfasuxidine alone. However, the addition of a crude folic acid antagonist preparation to the pigs fed the same ration (basal + sulfasuxidine + folic acid — lot IIIB), the anti-pernicious anemia

liver preparation (lot IVB) and folic acid plus biotin (lot VB) proved to be quite effective in decreasing growth and efficiency of feed utilization. The pigs in lot I (basal) maintained approximately the same average daily gains throughout both phases of the experiment. During the second phase of this experiment the average daily gain of the pigs in lot IIA (basal + sulfasuxidine) was lower than that of the first phase by 0.20 pound. One pig in lot IIIB died 21 days after being fed the crude folic acid antagonist. On necropsy the pig showed the following gross pathology: spleen slightly enlarged, some renal hyperemia, and very extensive bronchopneumonia. The average daily weight loss of this lot of pigs was 0.19 pound. One pig in lot IVB died 3 days after being fed the folic acid antagonist. On necropsy this pig showed the following gross pathology: gross hemorrhage (whole blood) in both lungs, and peripheral infiltration of blood in spleen with swelling. The other pig in this lot gained only 2 pounds during the 8 weeks on this trial, or made an average daily gain of only 0.04 pound. It is difficult to state whether or not the deaths were due to the folic acid antagonist. However, the 2 pigs which died were the only death losses occurring during the experiment. Some deaths were reported in rats by Franklin et al. ('47) when this same crude folic acid antagonist was fed and insufficient folic acid was given.

In lot VA (basal + sulfasuxidine + folic acid + biotin) the gains were approximately equal to those of the pigs on the basal ration, showing that a combination of folic acid and biotin was effective in making up the decrease in growth caused by feeding sulfasuxidine in the ration, a finding in line with similar work obtained with the rat by Nielsen and Elvehjem ('42). The pigs in lot VB fed the same ration plus the folic acid antagonist gained at a slower rate than the pigs in lot VA. However, their average daily gains were greater than those of all lots of pigs except lots I and VA.

The biotin deficiency symptoms became much more pronounced during this second phase of the trial. The pigs in lot I did not, however, display any signs of a biotin deficiency.

Their hair coat was smooth and there were no cracks in their feet. The pig in lot IIA (basal + sulfasuxidine) developed some cracks in his feet and there was some hair loss on the lower and posterior hams. This showed that feeding sulfasuxidine over a long period of time will result in the appearance of slight symptoms of biotin deficiency. The pigs in lot IIB (basal + sulfasuxidine + antagonist) had only slight cracks in their feet, but their hair loss was slightly more pronounced with a dark exudate on the skin of 1 of these pigs. The pigs in lot IIIA (basal + sulfasuxidine + folic acid) displayed very severe biotin deficiency symptoms. Their feet were badly cracked and their hair coat was quite rough, the hair being sparse over the body and none was left on the hams. The pigs also had a small amount of brownish exudate throughout the surface of their body. The 1 pig still alive at the end of this trial in lot IIIB (basal + sulfasuxidine + folic acid + antagonist) had such badly cracked feet that it could hardly walk. The feet bled quite easily. The hair coat was rough, sparse and thin on the back and lower hams and the skin was covered with a dark exudate. The pigs in lot IVA (basal + sulfasuxidine + anti-pernicious anemia liver extract) also had badly cracked feet and walked with difficulty. Their hair coat was also rough. The hair on 1 pig was quite thin all over the body. Both pigs in this lot had a dark exudate on their skins. The 1 surviving pig in lot IVB (basal + sulfasuxidine + anti-pernicious anemia liver extract + antagonist) also had badly cracked feet. The results obtained during the second phase of this trial are in line with those in the first trial where the addition to the basal ration + sulfasuxidine of either folic acid or the anti-pernicious anemia liver extract increased the pigs' needs for biotin since the pigs exhibited very much more severe biotin deficiency symptoms. This is of considerable interest since it may suggest a possible need for biotin by human patients treated with folic acid or the anti-pernicious anemia liver extract. This statement takes on added significance due to the report of Heinle and Welch ('47) suggesting that the possibility needs to be considered that folic acid not

only fails to prevent but actually may precipitate neurologic relapse in some patients with pernicious anemia. At this station a spasticity of the hind legs has continually been observed in pigs with a biotin deficiency.

STUDIES OF THE BLOOD

The data contained in table 3 summarizes the results obtained in the blood studies with pigs during both phases of the experiment.

The data show that the basal ration, after being fed for 21 weeks, is not adequate for hemopoiesis in the pig. Red blood cell formation was more adversely affected than hemoglobin formation. A slight hyperchromic anemia developed. On the basis of these data and those obtained by Cunha et al. ('47) where folic acid stimulated hemoglobin formation with the same basal ration, it is recommended that folic acid be added to the basal ration containing the 6 B-complex vitamins. Of interest is the finding that adding alfalfa to the basal ration stimulated hemopoiesis to about the same extent as folic acid. Since alfalfa is a good source of folic acid, it may be that a good deal or all of the response is due to the folic acid in this plant.

The addition of sulfasuxidine to the basal ration for 21 weeks resulted in a moderate anemia being produced. Folic acid was very effective in preventing this anemia. It was more effective than the anti-pernicious anemia liver extract in preventing the anemia and in stimulating hemoglobin and red blood cell formation when sulfasuxidine alone was added to the basal ration. However, it must be realized that only 1 level of the anti-pernicious anemia liver extract was tried and that possibly a higher level may have given different results. A combination of folic acid and biotin was no better than folic acid alone in stimulating hemoglobin and red blood cell formation when sulfasuxidine was added to the basal ration.

The addition of a crude folic acid antagonist to the basal ration + sulfasuxidine resulted in a more severe anemia be-

TABLE 3
Blood picture of the pigs: hemoglobin (HB), erythrocytes (RBC) and leucocytes (WBC)

Lot No.	RATION	AV. HB ¹		HB		RBC		AV. RBC ¹		AV. WBC ¹		COLOR INDEX ² Assuming 12.5 gm Hb. and 7,000,000 RBC as being near normal
		First phase (13 wks.)	Second (8 wks.)	Low point during second phase	Low point during second phase	Low point during second phase	Low point during second phase	First phase (13 wks.)	Second (8 wks.)	First phase (13 wks.)	Second (8 wks.)	
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>Millions</i>	<i>Millions</i>	<i>Thousands</i>	<i>Thousands</i>	<i>Thousands</i>	<i>Thousands</i>	
I	Basal	10.80	10.88	9.57	4.76	5.66	20.0	23.4	1.12			
II	Basal + sulfasuxidine IIB: same as II + Antagonist	10.63	8.60	7.52	4.09	5.66	18.3	15.3	1.04			
III	Basal + sulfasuxidine + folic acid IIIB: same as III + Antagonist	12.71	14.42	13.04	7.21	7.50	22.0	24.4	1.00			
IV	Basal + sulfasuxidine + anti-pernicious anemia liver extract IVB: same as IV + Antagonist	11.57	10.91	10.52	5.27	5.81	22.3	18.5	1.12			
V	Basal + sulfasuxidine + folic acid + biotin VB: same as V + Antagonist	12.96	13.62	12.26	6.33	6.92	19.7	17.8	1.04			
VI	Basal + 10% alfalfa meal	12.87	11.18	9.80	5.79	5.87	25.5	22.6	0.95			
						6.49	19.8		1.12			

¹ Average figures indicate an average value obtained from all determinations conducted weekly.

² The color index is based on the low point in Hb and RBC during second phase of experiment, except in lot VI where the data from the first phase were used.

ing produced than when the sulfasuxidine alone was added to the basal ration. Folic acid was only slightly effective in preventing the effect of the antagonist, probably because it was not fed at a high enough level. When folic acid was fed in high enough levels it was able to counteract the effect of the same crude folic acid antagonist with the rat as shown by Franklin et al. ('47). The anti-pernicious anemia liver extract was not as effective as folic acid (at the level both were fed) in stimulating hemoglobin and red blood cell formation when the crude folic acid antagonist was added to the basal ration + sulfasuxidine. Of interest is the finding that a combination of biotin and folic acid was more effective than folic acid alone in stimulating red blood cell and hemoglobin formation when the crude folic acid antagonist was fed.

The number of red blood cells closely followed the level of hemoglobin in the blood. A normocytic anemia was produced in the pig which was prevented by folic acid, and to a lesser extent by an anti-pernicious anemia liver extract when sulfasuxidine was added to the basal ration. This is similar to the type of anemia produced by Cartwright et al. ('46) with the pig fed sulfasuxidine, which was cured with a liver extract. Differences in diets fed and the fact that it takes a long time for sulfasuxidine feeding to result in the development of anemia in the pig may account for the finding of Welch et al. ('47) that they were unable, by feeding sulfasuxidine to the pig, to produce an anemia, such as is reported in this paper and previously by Cartwright et al. ('46).

No leukopenia or decrease in granulocytes below normal occurred by adding sulfasuxidine or the crude folic acid antagonist to the basal ration. All pigs in lots III and IV showed some increase in granulocytes above the level obtained with the pigs in lot II. However, all the pigs in lot V showed very nearly the same level of granulocytes as occurred with the pigs in lot II. Where the granulocytes increased in the pigs in lots III and IV, there was a slight decrease in agranulocytes.

The use of the antagonist in lot IVB resulted in a definite hypochromic anemia. It is difficult to state definitely whether a folic acid deficiency results in a hypochromic or hyperchromic anemia since no hypochromic anemia developed when sulfasuxidine alone was added to the basal ration. This statement also needs to be qualified by stating that the color index used is based on 12.5 gm of hemoglobin and 7,000,000 RBC being regarded as near normal. If other values for "normal" were used, then the color index figures might be different and different conclusions might be drawn. On the other hand, if the color index in lot VI is taken as near normal, then a slight hypochromic anemia may have developed due to a folic acid deficiency. Anisocytosis was observed in all lots of pigs.

SUMMARY AND CONCLUSIONS

The basal ration, fed for 21 weeks, was inadequate for hematopoiesis in the pig. It is recommended that folic acid be added to the basal ration (containing 6 B-complex vitamins — thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, and choline) when it is used in studying the effects of other vitamins in the pig.

The addition of 10% dehydrated alfalfa meal to the basal ration, for a 13-week period, was of no benefit on growth or efficiency of feed utilization. However, it was beneficial to about the same extent as folic acid, in stimulating hemoglobin and red blood cell formation.

The addition of folic acid or the anti-pernicious anemia liver extract to the basal ration + sulfasuxidine resulted in an increased need for biotin since biotin deficiency symptoms showed up 2 weeks earlier and developed very much more severely than when only sulfasuxidine was added to the basal ration. This may have been caused by some vitamin imbalance. This might suggest the possibility of biotin therapy being needed for human patients being treated with folic acid or the anti-pernicious anemia liver extract. Sulfasuxidine feeding for 21 weeks resulted in the development of only slight symptoms of biotin deficiency.

A normocytic anemia was produced in the pig by adding sulfasuxidine for 21 weeks, to the basal ration. The anemia was prevented by folic acid and to a lesser extent by an anti-pernicious anemia liver extract (at levels both were fed). A more severe anemia was produced by using a crude folic acid antagonist. A combination of biotin and folic acid was more effective than folic acid alone in counteracting the effect of the crude folic acid antagonist.

The effect of sulfasuxidine, over a long period of time, in decreasing gains and efficiency of feed utilization was counteracted almost entirely by a combination of folic acid and biotin. However, sulfasuxidine did not affect gains or efficiency of feed utilization during the first 6 weeks the pigs were on experiment; neither did it affect hemoglobin or erythrocyte formation during the first 13 weeks on experiment. This indicates that sulfasuxidine can be used therapeutically, at levels fed in this experiment, for a considerable length of time without any visible injurious effect on the pig.

The anti-pernicious anemia liver extract had no effect (at level fed) in stimulating hemoglobin or erythrocyte formation when the crude folic acid antagonist was added to the basal ration + sulfasuxidine.

ACKNOWLEDGMENTS

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THE EFFECT OF ENZYMATIC HYDROLYSIS ON THE NUTRITIVE VALUE OF CASEIN

I. DIGESTION OF CASEIN WITH PANCREATIC ENZYMES

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ONE FIGURE

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Processing does at times have a marked effect on the nutritive value of dietary proteins. Heating certain proteins may reduce the nutritive quality presumably due to the formation of 'new linkages resistant to enzymatic digestion (Block et al., '46; Eldred and Rodney, '46), whereas, moderate heating of other proteins like soybean may increase the nutritive value presumably through facilitating subsequent liberation of methionine during the digestive process (Melnick, Oser and Weiss, '46).

The latter observation, then, raises the question whether or not preliminary enzymatic digestion, before feeding, will alter the rate of absorption of certain of the contained amino acids and thereby change the nutritive value. The data available favor the view that the hydrolysis of a protein reduces, rather than enhances, the nutritive value by destroying some essential constituents. Miller, Robscheit-Robbins and Whipple ('47), for example, suggest that some unidentified substances present in the dietary proteins and absent in mixtures of amino acids are responsible for maintenance of weight in depleted dogs. These unidentified factors may be similar to those reported by Wonnack and Rose ('46) who have pre-

sented evidence that protein may contain substances which are required for maximum increases in weight, substances not found in amino acid mixtures or certain protein digests. The recent work of Woolley ('41, '46) demonstrates that one or more essential peptides may be furnished by the intact protein and not by a complete hydrolysate, and the absence of these may lower the nutritive value of the hydrolysate.

It was for these reasons that the relationship between the degree of enzymatic hydrolysis and the nutritive value of a protein was studied. The experiment reported here was undertaken to determine the effect of enzymatic predigestion of casein on the nutritive value of the protein. Casein was selected for this study because (a) of the ease of its digestion by pancreatic enzymes and (b) of its high nutritive properties which have been carefully characterized. Digestive enzymes were used to catalyze hydrolysis simulating the type of digestion which takes place in the intestinal tract. The amount of enzyme used was small enough so that supplementation of casein with enzyme protein was not an important factor. Hydrolysates in which 18, 38 and 59% of the amino acids were liberated, were prepared. The nutritive values of casein and these 3 hydrolysates were then determined by measuring their nitrogen balance indexes in adult dogs and their protein efficiencies in rats.

THE ENZYMATIC STUDIES

In order to minimize a supplementation effect due to the addition of enzyme amino acids, the enzymes of crude extracts of beef or hog pancreas were fractionated and the enzymes activated. The procedure utilized is given below.

Purification and activation of tryptic enzymes

One hundred and fifty grams of freshly ground beef or hog pancreas were extracted in a Waring Blendor for 5 minutes with 290 ml of water and 12.5 ml of 6 N H_2SO_4 . The pH of the suspension was raised to 4.2 with 10 ml of 6 N NaOH, and the

mixture was filtered. In order to activate the enzyme the pH of the filtrate was raised to about 7.8 and kept at 0°C. (ice water mixture) for approximately 72 hours. This affected an increase in enzyme activity from 17¹ to approximately 770 units per milliliter and 550 units per milligram of nitrogen. The time necessary for activation can be reduced if a sample of previously activated enzyme solution is added in an amount equivalent to 5% of the total enzyme activity. A further purification is possible through precipitation of enzyme at 0°C. with ethyl alcohol (70%). The unit activity of such alcohol-precipitated enzyme is about 1700 units per milligram nitrogen instead of 550. Crystalline trypsin and crystalline chymotrypsin contain 800 and 1700 units per milligram nitrogen, respectively, according to the test method utilized.

Preparation of carboxypeptidase

Crystalline carboxypeptidases were prepared according to the procedure described by Anson ('37).

Preparation of casein hydrolysate solutions

(1) *18% Hydrolysis.* The procedure for digestion is briefly as follows: Eight hundred grams of crude edible casein were suspended in 8 l of distilled water and enough sodium hydroxide (approximately 104 ml of 4 N NaOH) added to bring the pH of the solution to about 7.6. Five grams of the purified tryptic enzymes were added to the solution and the digest kept at 55°C. Aliquots were taken periodically to follow the course of digestion, using the increase of amino nitrogen by formol titration as a criterion. It was found that the digestion reached a maximum of 18% in less than 24 hours. Further addition of purified enzyme at this point did not materially

¹One unit of tryptic activity is defined as the amount of enzyme which will digest 1 ml of 0.25% casein solution at pH 7.6 and 37.5°C. to the extent of 50% in 15 minutes. The details of the method will be published in the J. Gen. Physiology, 1948 (B. F. Chow and M. W. Peticolas).

increase digestion. After maximum digestion, the whole suspension was lyophilized without filtering in order to include in the final preparation all essential amino acids and/or accessory growth factor. The solid material (about 900 gm) was used for biological and chemical analysis. The material contained 13.2% total nitrogen, of which 18% was amino nitrogen by the ninhydrin method.

(2) *40% Hydrolysis*. In order to obtain a 40% hydrolysis, a highly purified carboxypeptidase was added to the 18% casein hydrolysate as prepared under (1). Approximately 3 gm of purified carboxypeptidase were added to each 800 gm of the 18% hydrolysate. The degree of digestion is also followed by the formol titration, and after maximal digestion the suspension was lyophilized. The solid material was found to contain 13.5% nitrogen, 40% of which was alpha amino nitrogen by the ninhydrin method.

(3) *60% Hydrolysis*. A solution of 800 gm of crude casein was made in the usual manner. Four grams of purified hog pancreatic extract which contained, besides the tryptic enzymes and carboxypeptidases, amino polypeptidase were added and the digestion was allowed to proceed at 55°C. for 48 hours; the mixture was then lyophilized. The dry powder was found to contain 13.5% total nitrogen, of which 60% was alpha amino nitrogen by the ninhydrin method.

THE BIOLOGICAL ASSAYS

Protein efficiencies in rats were determined by the Osborne-Mendel method ('26). Fifteen rats were used for each test hydrolysate at the following nitrogen levels in the diet: 1.1%, 1.4% and 1.7%. The nitrogen balance indexes in dogs (K) were calculated according to the following equation:

$$NB = K(AN) + NE.$$

where NB is nitrogen balance, AN is absorbed nitrogen and NE, is the excretion of nitrogen on a protein-free diet (Allison, Anderson and Seeley, '46). Seven dogs were fed a protein-free diet for 8 days. The urine and feces samples were

collected the last 3 days to determine NE_0 . Each dog was then fed the casein sample at a level of approximately 0.15 gm nitrogen per kilogram body weight per day for 8 days. The first 2 days were an adjustment period after which two 3-day urine samples were collected. Each dog returned to the protein-free diet for 5 days with a 2-day adjustment period and a 3-day urine and feces collection period. Thus, duplicate measurements were made on each dog with an NE_0 associated with each sample. These same dogs were then returned to the stock laboratory diet for 2 to 3 weeks and used for another test.

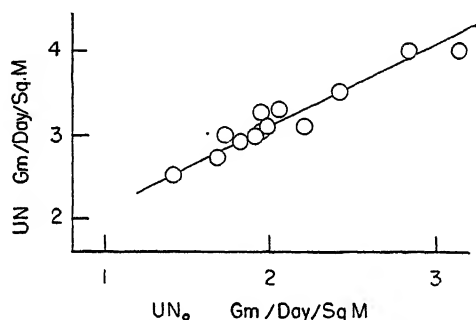


Fig. 1 Urinary nitrogen excretion in dogs fed casein nitrogen against excretion of nitrogen on a protein-free diet.

Previous studies have demonstrated that if absorbed nitrogen (AN) from any one protein is constant, the following relationship should hold:

$$UN = UN_0 - C$$

where UN is the urinary nitrogen excretion during nitrogen feeding and UN_0 is the urinary nitrogen excretion while the dogs are receiving a protein-free diet (Allison, Seeley and Ferguson, '47). The value of C is a constant equal to $(1-K)(AN)$ where K is the nitrogen balance index. On the other hand, if there has been some shift in the physiological state of the dog, the values for K will no longer be constant. Data obtained while feeding casein to the dogs in the studies reported here are illustrated in figure 1. The relationship

between UN and UNo is linear with a slope of unity. The constant C is equal to 1.1, and AN is 3.45 gm/day/sq. M. The nitrogen balance index K is equal therefore to 0.68.

Average values for nitrogen balance indexes for casein and for the hydrolysates fed to dogs are recorded in table 1, together with the protein efficiency measurements in the rats. The data demonstrate that predigestion of casein with the enzymes does not alter the utilization of the nitrogen as measured by balance indexes and protein efficiencies. These results can be interpreted to mean that hydrolysis did not

TABLE 1

The effect of degree of hydrolysis on the nutritive properties of casein

DEGREE OF HYDROLYSIS	DIGESTIBILITY IN DOGS	NITROGEN BALANCE INDEX IN DOGS	PROTEIN EFFICIENCY IN RATS	STREPOGENIN CONTENT	
				Actual	Potential
%	%			<i>Units/gram</i>	
0	98 ± 0.8 (7) ¹	0.68 ± 0.05	2.3 (15) ¹	0	5
18	98 ± 1.0 (7) ¹	0.67 ± 0.04	2.2 (15) ¹	1	5.5
38	98 ± 1.0 (7) ¹	0.67 ± 0.05	..	5	5.5
53	97 ± 1.8 (7) ¹	0.69 ± 0.08	..	5	5
59	95 ± 1.7 (6) ¹	0.68 ± 0.06	2.4 (14) ¹	5	5

¹ The number inside the parentheses indicates the number of animals used for each determination.

change the nutritive value of the protein. It should be emphasized that ingested casein is rapidly digested by enzymes of the gastro-intestinal tract so that no matter what the preliminary hydrolysis may have been, the liberation of essential amino acids in the gut may be from whole protein and from an hydrolysate quite similar.

DISCUSSION

These observations do not rule out the possibility that complete hydrolysis of casein would alter the retention of nitrogen in the animal by changing the pattern of amino acid absorbed. It is commonly reported and assumed that complete hydrolysis reduces the biological value of a nitrogen source (Supplee and

Clark, '46). These reports are based, however, on feeding hydrolysates made with acid or by feeding mixtures of amino acids. Acid hydrolysis destroys tryptophane and possibly other amino acids which must be restored, and restorations may not always be adequate. Furthermore, the lower biological values observed during the feeding of amino acid mixtures may be associated with the presence of unnatural isomers (Albanese and Irby, '43; Murlin et al., '46). Cannon et al. ('46), on the other hand, found that a mixture of 16 amino acids patterned after casein produced gains in protein-depleted rats equivalent to that yielded by casein itself. Under the conditions of the experiments reported here, however, the hydrolysates reached the gut with certain polypeptides intact, making possible their absorption or their hydrolysis by peptidases in the normal manner. The potential streptogenin content of the casein, for example, was not altered by the enzymatic hydrolyses (see table 1). Thus it would appear that enzymatic hydrolysates, at least of casein, prepared according to methods described in this paper, retain the nutritive properties of the parent protein.

SUMMARY

Casein was hydrolyzed to different degrees (18%, 40%, 60%) through the selective use of pancreatic enzymes under conditions which minimize any destruction of amino acid patterns. It was found that the nitrogen balance indexes and the protein efficiency values of the hydrolysates remained essentially identical with those of the undigested protein.

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THE EFFECT OF ENZYMATIC HYDROLYSIS ON THE NUTRITIVE VALUE OF CASEIN

II. DIGESTION OF CASEIN WITH BACTERIAL AND FUNGAL ENZYMES

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ONE FIGURE

(Received for publication March 18, 1948)

Woolley ('41) reported that streptogenin, a growth nutrient present in casein, is liberated by the digestion with trypsin but not with pepsin, although the latter carries the hydrolysis to a greater extent. These results led to the suggestion that the position of cleavage of the protein molecule may be a more important factor in determining the nutritive value of a hydrolysate than the degree of hydrolysis. The stepwise hydrolysis of casein (Chow, Allison and White, '48) by purified pancreatic enzymes did not alter the nitrogen balance indexes or the protein efficiencies of hydrolysates as compared to the undigested protein. However, hydrolysis at different positions would yield peptides with different amino acid patterns. This can be done by using enzymes which are not present in the digestive tracts of mammalian experimental animals. This plan was utilized in order to determine whether such predigestion would affect the nutritive value of the resulting hydrolysate.

¹ The author acknowledges the technical assistance of Claire Federbush and Harry Slocum in the nitrogen balance determinations.

EXPERIMENTAL²*(1) Enzymes used*

Two commercially available enzymes (Rhozyme P-11³ isolated from fungus and Protease 15⁴ isolated from bacteria) were chosen for this study because they were found to split the protein chain of the casein molecule at different positions.

(2) Digestion of casein

The procedure for digestion was as follows: Eight hundred grams crude edible casein⁵ were suspended in 8 l of distilled water and enough sodium hydroxide (approximately 104 ml of 4 N NaOH) added with efficient stirring to bring the pH of the solution to about 7.6. Ten grams of the enzyme powder, either Protease 15 or Rhozyme P-11, containing approximately 6% nitrogen were then added to the solution incubated at 37°C. An additional portion of the enzyme (2.5 gm) was added on the third day and the digestion was allowed to continue for 4 additional days. Aliquots were taken periodically to follow the course of the digestion with the formol titration method. After the maximal digestion, the whole suspension was lyophilized, in order to minimize the loss of essential amino acids or growth factors that might be contained in an insoluble residue. The yield of the hydrolysate was 850 gm.

(3) Determination of nitrogen balance index

The procedure for the determination of nitrogen balance index is essentially that of Allison, Anderson and Seeley ('46) and has been described in detail in the preceding paper.

² The author acknowledges the technical assistance of Lois Barrows for the preparations of hydrolysates.

³ Rhozyme P-11 obtainable from Rohm and Haas Company, Philadelphia, Pa.

⁴ Protease 15 obtainable from Rohm and Haas Company, Philadelphia, Pa.

⁵ The Borden Company, New York City, N. Y.

RESULTS

(1) Hydrolysis of casein by different enzymes

If 2 enzymes have sufficient specificity to attack a protein molecule at different points, the addition of the second enzyme to the protein solution previously digested to completion by the first enzyme, should cause a demonstrable further

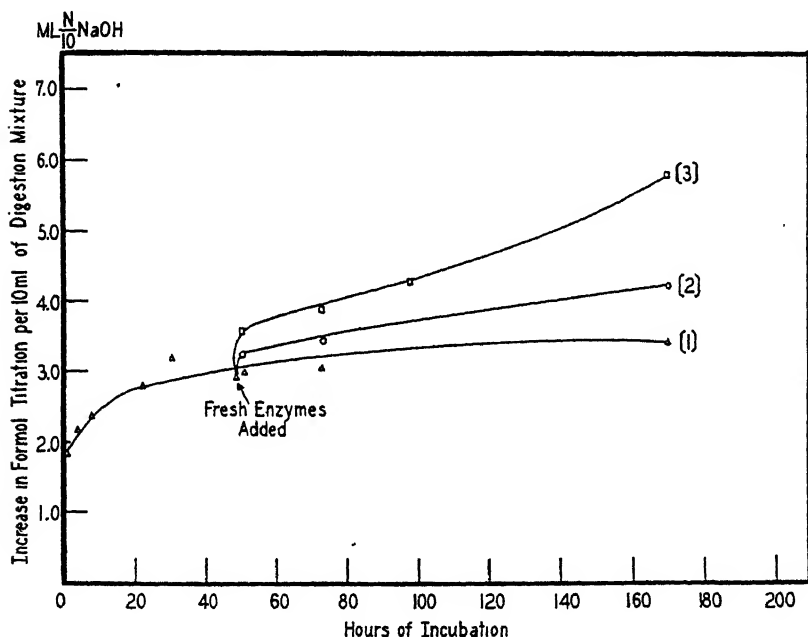


Fig. 1 The effect of addition of Rhozyme P-11 (curve 3) and of Protease 15 (curve 2) on the digestion of a casein previously hydrolyzed by purified pancreatic enzymes (curve 1).

digestion. The same situation should obtain if one reverses the order of addition of enzyme. This increase can be most conveniently followed by the formol titration method which was used to demonstrate that the 2 proteolytic enzymes isolated from bacterial or fungal origins split casein at different parts of the molecule. An example of the experimental data which demonstrate this difference is summarized in figure 1.

It can be noted in figure 1 that the addition of Protease 15 or Rhozyme P-11 to casein previously digested with purified pancreatic enzymes to the maximum extent of 18% (Chow, Allison and White, '48), causes a further increase in alpha amino acid nitrogen. Similar curves were obtained upon the addition either of purified pancreatic enzymes or of Rhozyme P-11 to a casein solution previously digested by Protease 15, or upon the addition of either Protease 15 or purified pancreatic enzymes to the Rhozyme digests of casein.

(2) *Determination of nutritive values*

Nitrogen balance index:

The nutritive values of casein and its hydrolysates were determined by measuring their nitrogen balance indexes and

TABLE 1
The nitrogen balance index of casein and its hydrolysate

EXPERIMENT NUMBER	SUBSTRATE	DIGESTION %	NUMBER OF ANIMALS	AVERAGE K + STANDARD ERROR
1	Casein	0.0	9	0.77 \pm 0.03
2	Protease digest	18	9	0.73 \pm 0.02
3	Rhozyme digest ¹ (1)	40	7	0.77 \pm 0.03
4	Rhozyme digest (2)	40	4	0.79 \pm 0.05
5	Rhozyme digest (3)	40	5	0.72 \pm 0.03

¹ Rhozyme digests (1), (2) and (3) were made on 3 separate batches.

their protein efficiencies. The former measures the amount of a protein or a hydrolysate required for the maintenance of nitrogen equilibrium in adult dogs. The latter measures the amount of a protein or of a hydrolysate required for the normal growth of weaned rats.

The results of the nitrogen balance studies (see table 1) indicate that this sample of casein and its 2 hydrolysates have the nitrogen balance indexes of 0.77 (casein), 0.72 to 0.79 (Rhozyme digest) and 0.73 (Protease digest), respectively.

These slight differences are found to be statistically insignificant (Fisher and Yates, '43).⁶

For the determination of protein efficiency, 3 groups of ten 23-day-old rats were fed casein or one of the hydrolysates as the sole source of amino acid nitrogen at the following nitrogen levels in the diet; 1.1%, 1.4% and 1.7%. The weights of individual animals as well as the food consumption were recorded twice a week. The maximum ratios of weight gain to food consumed, i.e., protein efficiency, after 3 weeks of feeding at 1.4% nitrogen level are 2.2 for casein, 2.0 for Rhozyme digest and 2.0 for Protease digest. Therefore, there appears to be no significant difference in the growth promoting properties of the protein or the hydrolysates.

The failure to demonstrate any difference in nutritive qualities measured either in terms of nitrogen balance indexes or in protein efficiency may be taken to mean a lack of appreciable destruction or removal of any limiting essential amino acids or polypeptides necessary for maintenance or for growth requirements either during the digestion or during the lyophilization of the hydrolysates. It also indicates that the amount of nitrogen in the added enzyme was of such a small order of magnitude as not to materially change the amino acid patterns of the hydrolysates.

The presence of an adequate amount of streptogenin or some yet unidentified factor has been shown to be necessary for optimal rat growth (Woolley, '41; Womack and Rose, '46). There were only 2 units of streptogenin⁷ per gram of the Protease digest, 5 units per gram of the Rhozyme digest and none in casein itself. The digestion of casein or Protease di-

⁶ It should be pointed out that there is a slight but significant difference in the nitrogen balance indexes, reported in this and the preceding paper. They were determined on the same sample of casein, but with 2 sets of dogs kept in 2 separate laboratories and on different preparatory diets before determination. Whether this difference in the index values can be explained on the animal variations alone, is still not certain at this time. This, however, does not invalidate our conclusions since the comparisons of the protein and its respective hydrolysates were performed on the same set of animals.

⁷ The author is indebted to Dr. A. Black, of E. R. Squibb & Sons, for the streptogenin determinations.

gest with trypsin liberated 5 units and 3 additional units, respectively, but the strepogenin content of the Rhozyme digest was not influenced by the same treatment.

These experiments provide confirmatory evidence that the 3 enzymes attack different parts of the casein molecule. They demonstrate that neither the position, the extent of cleavage of the casein molecule, nor the type of enzymes used has influenced the nutritive properties of the hydrolysate, even though in one case the content of the growth factors like strepogenin has been liberated but not destroyed.

SUMMARY

Casein was digested by Protease 15 and Rhozyme P-11 under conditions which minimized any change from the original amino acid composition of casein either by the removal or destruction of amino acids or by the addition of any significant amounts of amino acids from the enzymes. When fed to rats and dogs, the 2 hydrolysates prepared by the digestion of casein in neutral medium were found to yield the same nitrogen balance indexes and protein efficiencies as the whole protein.

These results suggest that neither the degree of digestion up to 40% nor the mode of attack necessarily affect the nutritive values of casein hydrolysates, so long as essential amino acids and perhaps the growth factors in casein are not destroyed.

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STUDIES IN AMINO ACID UTILIZATION

III. THE ROLE OF THE INDISPENSABLE AMINO ACIDS IN MAINTENANCE OF THE ADULT ALBINO RAT ¹

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ONE FIGURE

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INTRODUCTION

Because of conflicting evidence reported by 2 groups of workers, uncertainty exists concerning the role of some of the "essential" amino acids for maintenance of the adult white rat. Thus, 1 group (Wolf, '38; Wolf and Corley, '39) has reported that, except for arginine, all the amino acids essential for growth in young rats are essential, also, for maintenance of nitrogen equilibrium in adult rats. Another group using similar animals, rations and techniques (Burroughs, Burroughs and Mitchell, '40) has concluded that only 5 of the 10 amino acids essential for growth, viz., tryptophane, threonine, isoleucine, valine and methionine, are essential for maintenance of nitrogen balance in the adult rat.

¹The research which this paper reports was undertaken in cooperation with the Navy Department Office of Naval Research and the Committee on Food Research of the Quartermaster Food and Container Institute for the armed forces. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

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Other workers, however, have found that 8 or 9 of the amino acids essential for growth of the rat are also necessary for the maintenance of nitrogen equilibrium in the dog (Rose and Rice, '39) and man (Rose et al., '43). In view of these findings one would not expect that 3 of the amino acids essential both for growth in the rat and for maintenance of nitrogen balance in man or dog, viz., lysine, leucine and phenylalanine, should be nonessential for maintenance of nitrogen balance in the adult rat. More recently, Neuberger and Webster ('45) have shown that at least 1 of these, lysine, is essential for the maintenance of body weight as well as liver protein and concentration of blood protein in the adult rat. Mitchell ('47) has criticized this work because *ad libitum* feeding was used and has suggested that the results may indicate inadequate caloric intake rather than the indispensability of lysine. He has offered an hypothesis to explain the apparent species difference in the essential amino acid requirements for maintenance between man (or the dog) and the rat. Because the rat grows at a much faster rate than man or the dog, he suggests that this species might retain in maturity a greater capacity to synthesize certain of the essential amino acids. He believes that this synthesis might be adequate to meet the animal's maintenance needs.

We undertook this study because of earlier findings in the adult protein-depleted rat that, with the exception of arginine, all the essential amino acids were necessary for effective protein-repletion when fed in a ration adequate in other dietary constituents and containing a mixture of purified amino acids as the sole source of dietary nitrogen (Frazier et al., '47; Benditt et al., '47). For example, whenever any one of the 9 essential amino acids was omitted from the ration the animal promptly lost appetite and weight and failed to regenerate serum protein or hemoglobin. This trend was quickly reversed, however, when the missing amino acid was added to the ration. In the following study the response of the normal adult rat to certain amino acid deficiencies was investigated in a similar manner. In addition, the nitrogen

balance technique and force-feeding method were used in some experiments. The results indicate that for maintenance of the adult male rat all the amino acids now known to be essential for growth are required, with the possible exception of arginine.

EXPERIMENTAL METHODS

The rations used were patterned after those employed in former experiments in this laboratory with protein-depleted rats (Frazier et al., '47). These will be referred to hereafter as rations A, B, and C. Ration A contained a mixture of 16 purified amino acids; ration B, the 10 essential amino acids; and ration C, the essential amino acids minus arginine (table 1). For further details concerning composition of the diets, sources and purity of the amino acids, and the method of omitting an amino acid from the ration, the reader is referred to Frazier et al. ('47). The quantities of each amino acid contained in the daily allotment of the basal diets employed by Wolf and Corley ('39) and Burroughs, Burroughs and Mitchell ('40) are also recorded in table 1 for purposes of comparison. It should be noted that the proportions of the various amino acids in our rations were modeled after those in casein whereas the previous workers used mixtures which in most instances contained an amount of each amino acid equivalent to 4 mg of nitrogen daily.

Sixty-three male albino rats, of the Sprague-Dawley strain, weighing from 262 to 325 gm, and approximately 5 months of age were used. Prior to the beginning of the experiment the rats had been fed a ration containing 22% casein (3C), for approximately 3 months (Wissler et al., '45). Before each experiment was started initial weights were obtained after a 12-hour fast. In the experiments in which rations lacking an essential amino acid were tested, pairs of rats of comparable weights were used, 1 being fed ration A and its partner this ration devoid of the amino acid in question. In most instances both rations were fed for 20 days, during which time

Amino acid mixtures employed in maintenance experiments

AMINO ACIDS	MG OF AMINO ACID PER RAT PER DAY AS ADDED TO RATION						MG PER RAT PER DAY OF UTILIZABLE ESSENTIAL AMINO ACIDS ¹						
	Form	Diet A	Diet B	Diet C	Form	Wolf and Corley Ration no. 43	Form	Burroughs et al. Basal Ration	Diet A ²	Diet B ²	Diet C ²	Wolf and Corley Ration no. 43	Burroughs et al. Basal Ration
Arginine	$\left\{ \begin{array}{l} \text{HCl,} \\ \text{L} \end{array} \right.$	76.7	119.9	.	.	.	HCl, L	15.0	63.4	99.1	.	.	12.4
Histidine	$\left\{ \begin{array}{l} \text{HCl,} \\ \text{H}_2\text{O} \end{array} \right.$	52.0	81.6	87.4	$\left\{ \begin{array}{l} \text{HCl,} \\ \text{H}_2\text{O} \end{array} \right.$	20.0	HCl, H ₂ O DL	20.0	38.5	60.4	64.7	14.8	14.8
Isoleucine	DL	200.0	314.4	336.6	L	37.4	DL	37.4	100.0	157.2	168.3	37.4	18.7
Leucine	L	190.0	294.3	313.1	L	37.4	DL	37.4	190.0	294.3	313.1	37.4	18.7
Lysine	$\left\{ \begin{array}{l} \text{HCl,} \\ \text{H}_2\text{O} \end{array} \right.$	145.5	229.6	245.1	2HCl L	31.4	HCl DL	26.1	106.0	167.2	178.5	20.9	13.0
Methionine	DL	54.0	84.6	90.6	DL	42.6	DL	42.6	54.0	84.6	90.6	42.6	42.6
Phenylalanine	DL	80.0	125.7	134.6	L	47.2	DL	47.2	80.0	125.7	134.6	47.2	47.2
Threonine	DL	120.0	188.6	201.8	Synthetic	113.4	DL	34.0	60.0	94.3	100.9	28.4	17.0
Tryptophane	DL	27.7	43.5	46.6	L	24.3	L	29.2	27.7	43.5	46.6	24.3	29.2
Valine	DL	216.0	338.4	362.4	L	33.4	DL	33.4	108.0	169.2	181.2	33.4	16.7
Alanine	DL	86.5	DL	25.4
Aspartic acid	DL	97.0	DL	38.0
Cystine	L	5.6	L	24.3
Glutamic acid	L	366.5	DL	47.2
Glycine	.	7.7	H ₂ O	21.4
Tyrosine	L	99.0	L	51.7
Serine	DL	30.0
Norleucine	DL	37.4
Proline	L	32.9
Hydroxyproline	L	37.4

¹ For purposes of calculation of utilizable essential amino acids the D forms of tryptophane, phenylalanine, methionine and histidine have been assumed to be utilizable.

² The amino acids listed for ratios A, B and C represent the quantities in 15 gm of each ration (with the exception of the high fat ration where they represent the quantities in 9 gm of ration). These figures used with the diet consumption data listed in tables 2 to 5 make it possible to calculate individual amino acid intakes for any period.

the daily food consumption was recorded and the rats were weighed at fixed intervals. In some experiments the rations were interchanged at the end of the 20-day period so that the rat which had received the deficient ration was offered the complete one, and vice versa, for an additional 10 days. When nitrogen balances were determined, however, it was always during the first 14 days of the feeding period.

Nitrogen balance determinations

Two consecutive 7-day nitrogen balances measured for each rat receiving an amino acid ration served as mutual checks. In each instance these were preceded by a preliminary collection period of 7 days during which the animal was fed a casein ration which furnished 216 mg of nitrogen daily (Frazier et al., '47). Urine for each period was collected on filter paper impregnated with boric acid. Feces and waste food (the latter usually minimal) were collected separately each day. The boric acid papers were rinsed free of their nitrogen with 1% sulfuric acid. Duplicate samples of urine and feces were then analyzed for nitrogen by the Kjeldahl method. The net nitrogen intake for each period was calculated from the nitrogen content of the diet, multiplied by the amount consumed in 7 days from which the waste food had been subtracted. The nitrogen output for the week was the sum of the urinary and fecal nitrogen.

Force-feeding method

In 1 experiment (table 3) force-feeding via stomach tube was employed in order to equalize food intakes. The technique of Shay and Gruenstein ('46) was used and found to be satisfactory. The rats were fed thrice daily at approximately 5-hour intervals, using a special high-fat basal diet. This diet contained in 9 gm the same quantities of all constituents as were contained in 15 gm of ration A, but was concentrated by the substitution of fat for a calorically equivalent quantity of carbohydrate. In order to avoid any disturbance which

might result from overloading the gastrointestinal tract, the rats were given only 1.5 gm of ration per feeding the first day, 2.0 gm the second day and 3.0 gm the third day and thereafter. The ration was carefully weighed before each feeding, diluted with a little water and mixed in a small mortar, and then transferred quantitatively to a 10-ml syringe. After each feeding the syringe and stomach tube were rinsed with a small quantity of dilute sulfuric acid. At the end of the nitrogen balance period this pooled rinse water was analyzed for nitrogen and the amount subtracted from the dietary intake for the period.

EXPERIMENTAL FINDINGS

The role of the "nonessential" amino acids in maintenance was first determined in 6 rats (nos. 1-6 table 2), each rat being fed over the 20-day period the ration containing only the 10 essential amino acids (ration B) (Rose, '38). The results show that in every instance food consumption was good, weight was maintained, and nitrogen balance, when measured (nos. 3, 4, 5 and 6), was strongly positive. Therefore, the "nonessential" amino acids may be considered as dispensable, also, for maintenance of appetite, weight and nitrogen balance in the adult rat.

Six additional rats (nos. 7-12) were then fed ration C. This ration, containing only the 9 essential amino acids, appeared in some instances to depress appetite. In 3 of the 6 rats, weight losses of from 5 to 20 gm occurred in the 20-day period (table 2). However, food consumption with rats 11 and 12 was satisfactory and these animals maintained strongly positive nitrogen balances. In 3 of the 6 rats, moreover, weight was maintained or gains were recorded. Evidently, therefore, the absence of arginine exerts a somewhat variable effect upon weight and appetite. Nevertheless, the results obtained with rats 11 and 12 indicate that arginine is not necessary for strongly positive nitrogen balance and maintenance of weight when food consumption is satisfactory. Since it previously was shown that a lack of arginine did not interfere with weight

TABLE 2

Weight changes and nitrogen balances of adult well-nourished rats fed amino acid mixtures

ANIMAL NO.	RATION ¹	20-DAY PERIOD		NITROGEN BALANCE							
		Wt. gain or loss	Ration eaten	First 7 days				Second 7 days			
				N intake	Fecal N	Urinary N	N Balance	N intake	Fecal N	Urinary N	N Balance
		gm	gm	mg	mg	mg	mg	mg	mg	mg	mg
1	B	+ 8	294
2	B	± 0	298
3	B	+18	294	1617	159	1308	+150	1612	159	1278	+175
4	B	+11	295	1626	216	1228	+182	1628	180	1209	+239
5	B	+12	300	1645	236	1275	+134	1644	206	1304	+134
6	B	+23	320	1637	260	1114	+263	1637	270	1324	+ 43
7	C	-12	257
8	C	-20	239
9	C	- 5	288
10	C	- 1	286
11	C	+26	269	1352	165	834	+353	1253	212	716	+325
12	C	+15	282	1348	178	861	+309	1372	212	975	+185
13	A ²	+14	294	1564	135	1061	+368	1617	133	1167	+317
14	-Tryp.	-54	280	1478	235	1472	-229	1537	260	1549	-274
15	A ³	+ 6	298	1617	181	1113	+313	1617	159	1175	+283
16	-Meth.	-40	283	1534	214	1550	-230	1550	209	1530	-224
17	A ⁴	+13	295	1474	208	1170	+ 96	1500	178	1153	+169
18	-Iso.	-84	138	1068	101	1155	-188	804	154	1132	-717
19	A ⁵	+ 9	296	1465	180	1097	+188	1484	133	1124	+227
20	-Val.	-67	182	1006	121	1098	-212	1055	228	1240	-412
21	A ⁶	+ 5	296	1478	208	1078	+192	1485	193	1207	+ 85
22	-Threo.	-42	288	1460	235	1619	-394	1473	220	1692	-439

¹ The minus sign preceding the abbreviation of an amino acid indicates that this amino acid was omitted from ration A during this period.

² Identical with ration A except that it contained 13 mg of available tryptophane per daily portion.

³ Identical with ration A except that it contained 35 mg of available methionine per daily portion.

⁴ Identical with ration A except that it contained 65 mg of available isoleucine per daily portion.

⁵ Identical with ration A except that it contained 60 mg of available valine per daily portion.

⁶ Identical with ration A except that it contained 25 mg of available threonine per daily portion.

gain in the protein-depleted adult rat (Frazier et al., '47) where presumably the needs of tissue synthesis and maintenance must both be met, it would follow that it is not necessary for maintenance of the adult rat. The low urinary nitrogen output of the animals receiving ration C should receive further investigation.

We then studied the influence of a lack of tryptophane, valine, methionine, isoleucine and threonine, removed singly, upon weight, food consumption and nitrogen balance. Five pairs of normal adult rats were used with a slightly modified ration A serving as a control (see footnotes, tables 2 and 3). As can be seen, in every instance the lack of any one of these amino acids led to a marked loss of weight, and a negative nitrogen balance in each 7-day period. The effect upon food consumption, however, was variable. Thus, the rations lacking tryptophane, methionine and threonine led to less decrease in appetite than did those deficient in valine or, especially, isoleucine. The results leave little doubt, however, about the essentiality of each of these 5 amino acids for maintenance of the normal adult male rat, whether one measures maintenance of weight or of nitrogen balance.

Because lysine, leucine, histidine and phenylalanine were considered by Burroughs, Burroughs and Mitchell ('40) as non-essential for the maintenance of nitrogen balance, the effect of their dietary absence was next determined. The results were as follows: A dietary lack of lysine over the 20-day period led to a marked loss of weight in 4 rats tested (table 3), with a variable reduction in food consumption (from 2 to 26% in comparison with that of ration A). Although nitrogen balance was determined in only 1 rat, this was negative despite an almost complete consumption of the ration. Moreover, when the rations were interchanged for an additional 10-day period, weight losses of 11 and 17 gm were recorded for the 2 rats (23D and 24D) receiving the lysine-deficient diet, although the food consumption of these 2 animals equalled that of the 2 control rats which gained 22 and 14 gm, respectively, on ration A. When either histidine, leucine or phenyl-

TABLE 3

Weight changes and nitrogen balances of adult well-nourished rats fed amino acid mixtures

ANIMAL NO.	20-DAY PERIOD				10-DAY PERIOD				NITROGEN BALANCE							
	Ration ¹	Wt. gain or loss	gms	Ration eaten	Ration ¹	Wt. gain or loss	gms	Ration eaten	First 7 days				Second 7 days			
									N intake	Faecal N	Urinary N	N Balance	N intake	Faecal N	Urinary N	N Balance
23C	A	+11	284		-Lys.	-17	147		1369	167	924	+278	1360	194	1038	+28
24C	A	+10	285		-Lys.	-11	157		1324	212	1303	-136	1358	215	1190	-47
25C	A	+19	286						1469				1489	243	1102	+154
26C	A ^a	+1	291						1196	134	1075	-13	1204	220	1128	-144
23D	-Lys.	-30	223		A	+22	145		1477	194	1073	+210	1481	255	1037	+189
24D	-Lys.	-26	265		A	+14	185									
25D	-Lys.	-26	282						1235	204	1067	-36	1238	167	1215	-144
26D	-Lys.	-23	294						1525	209	1242	+63	1538	188	1124	+194
27C	A	+11	266		-Hist.	-19	100		1387	178	1379	-170	1335	202	1247	-114
28C	A ^a	+9	298		A	+27	144									
27D	-Hist.	-41	173		A											
28D	-Hist.	-46	211													
29C	A	+12	285		-Leuc.	-17	116									
30C	A	+6	273		-Leuc.	-26	100									
31C	A	+14	296													
32C	A ^a	-5	297													
29D	-Leuc.	-43	189		A	+18	143									
30D	-Leuc.	-45	202		A	+22	141									
31D	-Leuc.	-49	228													
32D	-Leuc.	-48	246													
33C	A	+2	288		-PA	-22	116									
34C	A	+9	293													
35C	A ^a	+8	297													
33D	-PA	-53	185		A	+15	149									
34D	-PA	-54	206													
35D	-PA	-28	243													

¹ The minus sign preceding the abbreviation of an amino acid indicates that this amino acid was omitted from ration A during this period. ^a Identical with ration A except that it contained 20 mg of available lysine per daily portion.

^a Identical with ration A except that it contained 15 mg of available histidine per daily portion.

^a Identical with ration A except that it contained 75 mg of available leucine per daily portion.

^a Identical with ration A except that it contained 30 mg of available phenylalanine per daily portion.

alanine was removed singly from ration A, both food consumption and weight declined; and during this time all animals tested were in negative nitrogen balance. When, however, the rations were interchanged, there was a prompt reversal in the pattern of food consumption and weight (table 3 and fig. 1). Thus, although the absence of lysine affected appetite and weight loss less severely than did the absence of the other 3 essential amino acids, there was little difference in the total negative nitrogen balance for the 14-day period, whether this was due to a deficiency of lysine, histidine or leucine. On the other hand, the absence of phenylalanine resulted in a more severely negative nitrogen balance but with less depression of appetite than did the absence of either histidine or leucine.

It is of interest that although a lack of a single essential amino acid in the ration affected appetite adversely in the well-nourished rat, it did so less severely than in the protein-deficient rat (Frazier et al., '47). Whereas the latter animal's appetite usually deteriorates within a day or two, in the normal well-nourished rat this effect may not manifest itself for 3 or 4 days (fig. 1). With both types of animal, moreover, the least effect upon appetite resulted from a deficiency of lysine.

In the work of Burroughs, Burroughs and Mitchell, it was reported that norleucine could substitute for either leucine or lysine, and tyrosine for phenylalanine. In our experiments this has not been true (table 4). For example, when norleucine was substituted for either leucine or lysine (rats 31S, 36S and 37S) there was a concomitant loss of appetite and weight, the weight loss being the same or greater than that of the animals fed a diet deficient in either lysine or leucine. The substitution of tyrosine for phenylalanine (rat 34S) also failed to prevent a loss of weight, although the weight decrease was somewhat less than it had been with phenylalanine deficiency alone (rat 34D). The latter observation is similar to the findings of others in growing rats (Rose and Womack, '46).

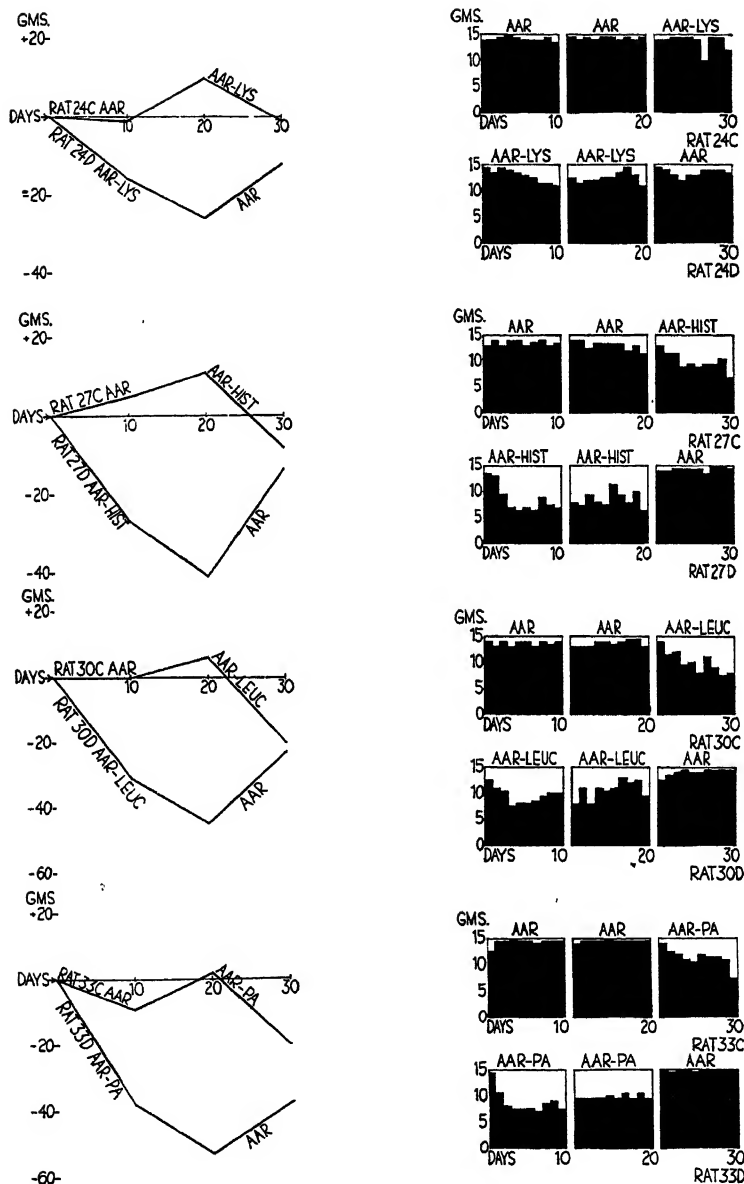


Fig. 1 Comparisons of weight changes and diet consumptions in adult normal rats fed ration A (AAR) and ration A devoid of 1 amino acid.

It is obvious that one cannot evaluate adequately the consequences of an acute amino acid deficiency until it has been shown that a similar effect will also occur with an adequate caloric intake. Therefore, experiments were performed in

TABLE 4

The effect of substituting norleucine for leucine, norleucine for lysine and tyrosine for phenylalanine upon weight and food consumption of normal adult rats

ANIMAL NUMBER	20-DAY PERIOD			10-DAY PERIOD		
	Ration	Wt. gain or loss	Ration eaten	Ration	Wt. gain or loss	Ration eaten
		gm	gm		gm	gm
31S	{ Nor- leucine for leucine	-59	207
36C	A	+27	290	{ Nor- leucine for lysine	-17	137
37C	A	+27	267		-14	120
36S	{ Nor- leucine	-27	209	A	+36	143
37S	{ for lysine	-18	233	A	+31	142
34S	{ Tyrosine for phenyl- alanine	-43	230

which the animals were force-fed, by stomach tube, rations devoid of lysine, leucine, histidine or phenylalanine (table 5). Pairs of rats were fed equal quantities of ration, one receiving the high fat ration A and the other this ration devoid of the particular amino acid being tested. It is apparent (table 5) that the removal of any one of these amino acids led to a loss of weight and a negative nitrogen balance, despite the comparable intake of all other dietary constituents. The negative nitrogen balances in each animal receiving a deficient mixture were associated with a substantial rise in urinary nitrogen as compared to the preliminary balance

period in which the animals consumed equal quantities of nitrogen from casein. It is of interest that the rat fed the histidine-deficient diet maintained a positive nitrogen balance for the first 7-day period but that during the second period it showed a net negative nitrogen balance. This is the only evidence we have obtained which indicates that histidine in the rat as in man (Rose et al., '43) may temporarily, at least, be dispensable for nitrogen balance.

TABLE 5

Weight changes and nitrogen balance data of adult well-nourished rats force-fed amino acid rations for 14 days

ANIMAL NO.	RATION	14-DAY PERIOD		NITROGEN BALANCE							
		Wt. gain or loss ¹	Ration eaten	First 7 days				Second 7 days			
				N intake	Urinary N	Fecal N	N Balance	N intake	Urinary N	Fecal N	N Balance
		gm	gm	mg	mg	mg	mg	mg	mg	mg	mg
38	A	+ 6	101.8	1159	969	103	+ 87	1348	1059	111	+188
39	—Lys.	—11	102.0	1093	1151	110	—168	1277	1276	118	—117
40	A	+ 6	107.3	1171	944	114	+113	1445	1007	106	+332
41	—Leuc.	—30	101.9	1121	1101	120	—100	1306	1449	94	—237
42	A	+15	102.4	1172	780	110	+282	1351	932	87	+332
43	—PA ²	—10	97.8	1160	1110	110	— 60	1326	1427	117	—218
44	A	+15	102.8	1168	885	124	+159	1352	1004	91	+257
45	—Hist.	—17	102.0	1144	1006	97	+ 41	1329	1343	115	—129

¹ Since all the animals lost weight during the first 2 days of the experiment in which they were being fed small amounts of ration the weight changes are calculated for the final 12 days of the experiment when the caloric intake was adequate.

² Phenylalanine.

DISCUSSION

What criterion should be used to measure the qualitative amino acid needs for maintenance of the adult animal? The answer to this question obviously depends upon how one defines "maintenance." In our study we have been primarily concerned with the maintenance of health, as evidenced by the preservation of weight and appetite as well as nitrogen

criteria of preservation of appetite and weight as well as nitrogen balance. A purified amino acid mixture patterned after the composition of casein served as the source of dietary nitrogen in the basal ration. The effects upon appetite and weight following removal of each of the 9 essential amino acids and all of the non-essential amino acids have been observed, as well as the effects of substituting norleucine for leucine or lysine and tyrosine for phenylalanine. When single amino acid deficiencies were studied, pairs of comparable rats, one receiving the complete ration and one the incomplete, were used. In some instances these rations were interchanged at the end of the first 20 days and the experiment was continued for an additional 10 days. Nitrogen balance determinations were made in representative animals during the first 14 days of the experiment, two 7-day collection periods serving as mutual checks. In 1 experiment the force-feeding technique was used to equalize the nitrogen and caloric intakes between the deficient and control rats.

The results may be summarized as follows:

1. The normal adult rat maintains appetite and weight when receiving rations in which only the 10 amino acids essential for growth are present.
2. The removal of arginine from this mixture produces a variable depression of appetite. When appetite is maintained, weight and a positive nitrogen balance are preserved.
3. The absence of each of the remaining 9 essential amino acids from the basal ration, although producing a variable depression in appetite, always leads to weight loss and a negative nitrogen balance. The absence of tryptophane, methionine and threonine does not produce the marked loss of appetite observed with the same deficiencies in the protein-depleted rat. The absence of lysine led to only slight interference with voluntary food consumption in both the protein-depleted and normal adult rat. In most instances the effect on food consumption was not quite so acute or marked as that observed previously in the protein-depleted rat.

4. Substitution of norleucine for leucine or lysine led to decreased appetite and weight loss similar to those produced by a deficiency of these amino acids. Substitution of tyrosine for phenylalanine resulted in a loss of appetite and weight somewhat less severe than that produced by phenylalanine deficiency.

5. Despite equalization of the food intakes of the rats deficient in lysine, leucine, histidine and phenylalanine with their controls by means of the force-feeding technique the animals on deficient rations lost weight and were in negative nitrogen balance.

6. Our results indicate that the same 9 essential amino acids required for growth of the young rat are necessary for maintenance of appetite, weight and nitrogen balance in the adult rat.

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BRAIN EXCITABILITY IN PYRIDOXINE-DEFICIENT RATS¹

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The spontaneous convulsive seizures of pyridoxine-deficient rats described by Chick, El Sadr and Worden ('40) and Daniel, Kline and Tolle ('42) offer 1 means for the study of the metabolic determinants of brain function. If the specific aberrations induced by pyridoxine deficiency can be correlated with abnormal behavior it can be concluded that processes involving pyridoxine are among the factors responsible for normal brain function. Accordingly, the work described below was done on rats which showed an increased brain excitability as a result of pyridoxine deficiency.

METHODS

Male rats obtained from the Sprague-Dawley Farm were used. A few of the animals were very young, weighing between 40 and 60 gm, but the majority weighed from 125 to 250 gm.

The vitamin B₆-deficient diet had the following percentage composition: sucrose 58, "vitamin-free" casein² 27, lard 5, hydrogenated cottonseed oil³ 5, salt mixture (Hubbell, Mendel and Wakeman, '37) 4, and cod liver oil 1. The following amounts of vitamins (in milligrams) were added to each kilogram: thiamine hydrochloride 20, riboflavin 30, calcium pan-

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² Labco.

³ Crisco.

tothenate 50, nicotinamide 100, inositol 100, p-aminobenzoic acid 600, 2-methyl-naphthoquinone 5, choline hydrochloride 500. In some cases the appearance of deficiency was hastened by the incorporation of 2,4-dimethyl-3-hydroxy-5-hydroxymethyl-pyridoxine (desoxyypyridoxine)⁴ in the diet. The desoxyypyridoxine was added to the extent of 10 or 50 mg to each kg of deficient diet. Identical results were obtained with the 2 concentrations in rats of the same size, but both were more rapidly effective in young than in adult rats. In other experiments, DL-tryptophane (10 gm/kg) or L-glutamic acid (20 gm/kg) was added. The control diet consisted of the basic diet with the addition of 50 mg pyridoxine hydrochloride/kg.

When given by subcutaneous injection, pyridoxine hydrochloride was administered as the unneutralized aqueous solution in a dose of 5 mg/rat. Xanthurenic acid was given by subcutaneous injection of a neutralized 0.1% aqueous solution.

Electroshock thresholds were determined as previously described (Davenport and Davenport, '48). The electroshock threshold is the smallest amount of current required to produce a detectable clonic seizure. Under rigidly controlled conditions its value, expressed in milliamperes (mA), is nearly constant from day to day but increases slowly in the normal rat from 10 mA at 50 gm to 20 mA at 250 gm.

Cellular hydration was accomplished by the method of Darrow and Yannet ('35). Isosmolar glucose solution, 10 ml/100 gm body weight, was injected intraperitoneally and was withdrawn 4 hours later, after it had been allowed to equilibrate with the extracellular fluid.

The usual qualitative test for xanthurenic acid in urine, described by Lepkovsky, Roboz and Haagen-Smit ('43), was employed. This consists of the development of a green pigment when a ferric salt is added to the urine and the pH is adjusted approximately to 8.

Blood for chemical analysis was removed from the inferior vena cava under intravenous pentobarbital anaesthesia.

⁴ The desoxyypyridoxine was generously supplied by Merck and Co.

Plasma was separated using the anaerobic technique of Davenport ('47). The skulls of the exsanguinated animals were opened, the brains removed, and the cerebral cortices separated from the major portion of the white matter. The water content of brain cortex and of plasma was determined by drying samples to constant weight at 105°C. Chloride was determined in plasma by the method of Van Slyke and Sendroy ('23) and in brain by the same method, using the Sunderman and Williams ('33) technique of alkaline digestion. A Perkin-Elmer flame photometer was used in the analysis of dry-ashed brain or plasma for sodium and potassium. The plasma pH was measured with a glass electrode, and the total carbon dioxide content was determined by the method of Van Slyke and Neill ('24).

Blood which was to be analyzed for lactic or pyruvic acid was withdrawn from the inferior vena cava with chilled syringes and measured at once into tubes containing the chilled precipitating reagents. The method of Barker and Summerson ('41) combined with the copper tungstate precipitation method of Somogyi ('31) was used in analyzing for lactic acid. Pyruvic acid was estimated by the method of Long ('42).

RESULTS

Vitamin B₆ deficiency and electroshock threshold

Table 1 is a summary of the effects of injected pyridoxine on the electroshock thresholds of control and vitamin B₆-deficient rats. The 6th and 8th columns show the mean change in electroshock threshold 5 and 24 hours, respectively, after the rats were given subcutaneous injections of pyridoxine hydrochloride. A comparison was made of the threshold of each rat before, 5 hours after, and 24 hours after the injection of pyridoxine, and the significances of the differences, calculated by Fisher's ('36) method, are given in columns 7 and 9.

The pair-fed controls of group 1 showed no change in electroshock threshold after administration of pyridoxine. The rats in group 2, which had no acrodynia and excreted no

xanthurenic acid after 5 to 6 weeks on the deficient diet, likewise showed no change after injection of the vitamin. In the moderately deficient rats of groups 3 and 4 there was a highly significant rise of threshold within 5 hours and a further rise

TABLE 1
Changes in electroshock threshold in rats receiving 5 mg pyridoxine

GROUP		ACRODYNIA	XANTHURENIC ACID	n ¹	CHANGE IN ELECTROSHOCK THRESHOLD			
					5 Hours		24 Hours	
					Change mA	P ²	Change mA	P ²
1	Controls	0	0	24	-0.1	0.4	-0.2	0.9
2	Not deficient after 5-6 weeks on deficient diet	0	0	7	-0.1	0.7	+0.1	0.8
3	Deficiency produced by basic deficient diet for 3-6 weeks	++	+++	20	+3.1	<0.01	+3.5	<0.01
4	Desoxy-pyridoxine for 15 days	++	+++	13	+2.2	<0.01	+3.0	<0.01
5	Desoxy-pyridoxine for 25 days	++++	+++	7	+1.3	0.2	+2.9	<0.01
6	Desoxy-pyridoxine for 5 days	0	++++	16	+0.2	0.1	+0.2	0.1

¹ Number of cases.

² Fisher, '36.

after 24 hours. The total rise amounted to 20% of the threshold before administration of pyridoxine. There was a slower but significant rise in threshold in the more severely deficient rats of group 5. In contrast to these, the rats in group 6 which had no evidence of deficiency except the excretion of high concentrations of xanthurenic acid showed no change after injection of the vitamin.

Table 2 summarizes the effects on electroshock threshold of tryptophane and glutamic acid added to the diet of rats. The mean change in electroshock threshold of each group is given in the 6th column. The 8th and 10th columns give the mean change in threshold 5 and 24 hours, respectively, after the administration of pyridoxine hydrochloride.

Each rat in groups 7 and 8 received slightly over 100 mg of DL-tryptophane daily. The addition of tryptophane to the deficient diet of group 7 resulted in a significant fall in the electroshock threshold. After the injection of pyridoxine there was a significant rise of threshold within 5 hours and a further rise by the end of 24 hours. On the other hand, the threshold of the control rats of group 8 failed to fall during the administration of tryptophane, or to rise in response to the injection of pyridoxine.

The addition of L-glutamic acid to the diet of mildly deficient rats appeared to raise the electroshock threshold. The animals of groups 9 and 10 were fed the basic deficient diet for 3 weeks, by the end of which time they showed signs of mild deficiency. To the diet of group 9 was added 2% of L-glutamic acid, while group 10 was continued on the basic deficient diet. By the end of 10 days the rats receiving glutamic acid showed a mean rise in electroshock threshold which was large enough to be significant, while the thresholds of the animals not receiving glutamic acid did not change. In more severely deficient rats the effect of glutamic acid was somewhat different. The 12 young rats of groups 11 and 12 were treated with desoxypyridoxine for 14 days. At the end of this time all had well-developed acrodynia and were excreting high concentrations of xanthurenic acid. Group 11 was then given glutamic acid in the basic deficient diet while group 12 was given the deficient diet alone. At the end of 6 days there was an insignificant drop in the electroshock threshold of both groups. The injection of pyridoxine then produced within 5 hours a highly significant rise of 2.5 mA in the threshold of group 11, whereas in group 12 it resulted in a rise of only 0.9 mA. Twenty-four hours after the injection of pyridoxine

the total rise in electroshock threshold was 3.3 mA in the animals which had received glutamic acid and 2.5 mA in the controls.

Cellular hydration and electroshock threshold

It has been shown by Swinyard, Toman and Goodman ('46) that cellular hydration reduces the electroshock threshold of normal rats about 56% in a regular and predictable manner. The same is true of rats having mild to moderate pyridoxine deficiency. In 10 pyridoxine-deficient rats the mean electroshock threshold was 17 mA before and 8 mA after cellular hydration. Six control rats had a mean threshold of 20 mA before and 9 mA after hydration.

Xanthurenic acid and electroshock threshold

In order to determine whether any of the results given above could be explained on the basis of convulsant properties of xanthurenic acid, the effect of subcutaneous injections of this substance upon the electroshock thresholds of rats was investigated.⁵ Ten normal rats were then given 1 mg of xanthurenic acid and 6 were given 2.5 mg in divided doses. At the end of 5 hours the electroshock thresholds were determined. The urines of the rats were examined for xanthurenic acid throughout the 5 hours and were found to be intensely positive within 1 hour; at the end of 5 hours the test in the urines of animals which received 2.5 mg was far more positive than was usually observed in the urines of our deficient rats. Although this would appear to indicate a higher blood concentration of xanthurenic acid than occurred in the pyridoxine-deficient rats, it produced no change in electroshock threshold. A single rat which was given a total dose of 5 mg of xanthurenic acid likewise showed no change in threshold, while another which received 10 mg had only a questionable slight lowering of the threshold.

⁵ An authentic sample of xanthurenic acid was synthesized for us by Dr. W. J. Horton of the Department of Chemistry, University of Utah.

Maximal seizure patterns

The pattern of maximal electroshock seizures has been described by Toman, Swinyard and Goodman ('46) as consisting of a latent period, a period of tonic flexion, a period of tonic extension, a clonic phase which is frequently absent, and a period of post-seizure depression. The average time from the application of the shock to the beginning of the extensor phase is 2.8 seconds and from the application of the shock to the end of the extensor phase, 14 seconds. The post-seizure depression is seldom less than 2 or more than 4 minutes in length.

In several respects the effect of pyridoxine deficiency on maximal seizure pattern is similar to that of anticonvulsant drugs. Moderate deficiency was produced in 11 young rats by the administration of desoxypyridoxine for 10 days. All these animals had shown normal seizure patterns before treatment with desoxypyridoxine. After treatment the latent period was increased slightly and the flexor phase was lengthened to an average duration of 8.5 seconds. The extensor phase was absent in 5 of the 11 rats and was extremely brief in the others. In all cases the clonic phase was present and the post-seizure depression was of normal duration. Injected xanthurenic acid had no effect on the maximal seizure pattern.

Brain and blood analyses

In order to determine whether the increased susceptibility of pyridoxine-deficient rats to electrically induced seizures may be explained on the basis of alterations in the electrolyte content of blood and brain, the analyses summarized in table 3 were made.

There was no difference between the results obtained on control and deficient rats. The data on both brain and plasma electrolytes are in essential agreement with those which Swinyard ('47) obtained in normal well-fed rats.

A few analyses were made to determine the acid-base balance in control and vitamin B₆-deficient rats. The blood sam-

ples in this case were not pooled. In 3 control and 3 deficient rats the acid-base picture was almost identical: the pH was 7.32 and the extra fixed acid amounted to approximately 6 mM/l. This low degree of acidosis, which in the control rats was undoubtedly the result of chronic starvation, does not account for any changes in electroshock threshold (Davenport and Davenport, '48).

The blood lactic and pyruvic acid levels were also the same in control and pyridoxine-deficient rats.

TABLE 3

Analyses of brains and plasma of control and pyridoxine-deficient rats

	WATER		CHLORIDE		SODIUM		POTASSIUM	
	n ¹	gm per kg	n	mEq per kg	n	mEq per kg	n	mEq per kg
Control brains	7	785	7	33.4	3	44.9	4	98.1
Deficient brains	7	786	6	33.4	4	45.0	4	98.0
Control plasma	5	921	4	106.1	4	145.8	1	5.2
Deficient plasma	4	920	3	105.4	3	147.0	1	5.1

¹ Number of pooled samples.

DISCUSSION

Growing rats which are given a diet deficient in vitamin B₆ show a progressive diminution in the rate of weight gain and a tendency for the electroshock threshold to fall. Weight-paired controls fed limited amounts of an adequate diet exhibit the same changes. The lowering of threshold in the controls is undoubtedly due to the fact that these animals are subjected to partial starvation (Davenport and Davenport, '48). In deficient rats starvation is probably not a major cause of the lowering of threshold, although it may be a contributory factor.

The injection of pyridoxine causes a rapid and highly significant rise of electroshock threshold in mildly deficient rats but has no effect in control animals fed appropriately limited amounts of an adequate diet. This indicates that even a mild degree of pyridoxine deficiency causes an increase in

brain excitability. In more severely deficient rats the injection of pyridoxine raises the threshold more slowly. Presumably in this case the changes caused by the deficiency are more profound and therefore less easily reversed. The effect of pyridoxine in correcting brain excitability of mildly deficient rats is not necessarily associated with increased food intake, for the 5-hour rise in electroshock threshold was observed in rats deprived of food for that interval. Reactions involving pyridoxine must therefore have some specific effect in the maintenance of normal brain excitability.

Our data provide both negative and positive evidence of the means by which a mild degree of pyridoxine deficiency alters the excitability of the brain. The deficiency does not change the electrolyte pattern of the brain or the response of the brain to the stress of cellular hydration; it does not change the acid-base or electrolyte pattern of the blood; and it does not change blood lactic or pyruvic acid concentrations. All these factors are known to cause or accompany increased brain excitability, and it is possible that they may be among the causes of the spontaneous convulsions of advanced pyridoxine deficiency, but their participation in the effects of early pyridoxine deficiency can be ruled out. On the other hand, the results of feeding DL-tryptophane and L-glutamic acid suggest strongly that an interference with transamination is responsible for the effect of pyridoxine deficiency on brain excitability.

The addition of extra tryptophane to the vitamin B₆-deficient diet intensifies the signs of deficiency, especially the excretion of xanthurenic acid (Lepkovsky, Roboz and Haagen-Smit, '43); Miller and Baumann, '45). Xanthurenic acid is a product of tryptophane metabolism, and its concentration in the urine is an index of the extent to which tryptophane metabolism has been deranged by the vitamin deficiency. The brain excitability of deficient rats increases when tryptophane is added to their diet. Our data show that this is not due to a convulsant action of the extra xanthurenic acid produced and suggest that it may not be directly concerned with de-

ranged tryptophane metabolism, since rats during the first few days of treatment with desoxypyridoxine excrete large amounts of xanthurenic acid but do not show a change in electroshock threshold. Rather, it seems probable that the presence of extra tryptophane increases the competition for pyridoxine among the enzyme systems which require this vitamin, and increases brain excitability indirectly, possibly through an effect on the transaminase system.

The data obtained from the experiments in which extra glutamic acid was fed to vitamin B₆-deficient rats present stronger positive evidence for a correlation between transamination and brain excitability. Glutamic acid is known to play a key role in transamination (Cohen and Hekhuis, '41). Although the administration of glutamic acid to rats maintained on a normal diet does not change their brain excitability (Goodman, Swinyard and Toman, '46), it raises the electroshock threshold of rats mildly deficient in vitamin B₆. In severe deficiency it fails to raise the threshold but does hasten its return toward normal after the injection of pyridoxine. Although it is true that the deficient diet itself contains about 6% of glutamic acid in the casein, it is possible that only a small percentage of this is released from the casein molecule and made available to the transaminase system. In any case, the result of adding 2% of free glutamic acid must be due to a mass action effect. A simple explanation of our findings is that extra glutamic acid promotes a more efficient utilization of pyridoxine in the transaminase system and that maintenance of normal transamination is essential for those tests of normal brain function employed in this study.

SUMMARY

1. The electroshock threshold of rats mildly deficient in pyridoxine rises significantly within 5 hours after the rats are injected with pyridoxine.
2. The electroshock threshold of weight-paired control rats is not affected by the injection of pyridoxine.

3. Cellular hydration reduces the electroshock threshold to a similar extent in normal and pyridoxine-deficient rats.

4. The concentrations of water, sodium, potassium and chloride in the brain and blood plasma and of lactic and pyruvic acid in the blood are the same in pyridoxine-deficient and control rats. Both types of rat have a very mild degree of metabolic acidosis.

5. When extra tryptophane is added to the diet of rats mildly deficient in pyridoxine the signs of deficiency are intensified and there is a fall in electroshock threshold. This fall can be reversed within 5 hours by the injection of pyridoxine.

6. Xanthurenic acid, a product of tryptophane metabolism, when injected into normal rats has no effect on electroshock threshold or maximal seizure pattern.

7. Feeding extra glutamic acid to rats mildly deficient in pyridoxine causes the electroshock threshold to rise.

8. Rats more severely deficient in pyridoxine show a slower rise in electroshock threshold after the injection of pyridoxine, but if the animals have been fed extra glutamic acid for 6 days they respond to injected pyridoxine as rapidly as do mildly deficient rats.

9. It is suggested that the maintenance of normal transaminase activity is essential for those tests of normal brain activity employed in this study.

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CATARACTS DUE TO DEFICIENCIES OF PHENYL- ALANINE AND OF HISTIDINE IN THE RAT. A COMPARISON WITH OTHER TYPES OF CATARACTS¹

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TWENTY-SEVEN FIGURES

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Cataracts which could be definitely attributed to a nutritional deficiency were first observed by Day, Langston and O'Brien ('31) in rats deficient in what was then known as vitamin G, although Salmon, Hays and Guerrant ('28) had earlier observed what might have been similar lenticular changes in studying experimental pellagra. It later became apparent that the cataracts were caused by a specific deficiency of riboflavin (Day, Darby and Langston, '37). Curtis, Hauge and Kraybill ('32) found that "rats receiving inadequate protein from either low corn protein or low protein of the basal ration in the absence of additions of tryptophane developed permanent blindness. This blindness was characterized by white opaqueness of the eye and loss of the characteristic colors of the eye, quite different from ophthalmia due to vitamin A deficiency." The addition of tryptophane to the diet prevented this blindness. From this description and the published photographs it seems apparent

¹ Preliminary reports of this work have been published (Sydenstricker, Schmidt and Hall, '47b; Bowles, Sydenstricker, Hall and Schmidt, '47).

that these authors had observed the cataracts resulting from tryptophane deficiency, as were later described by Totter and Day ('42).

That the adequacy of nutrition with respect to other amino acids and to protein may be of importance in the development of cataracts is indicated by several studies. Mitchell and Cook ('37) found that a protein deficiency definitely hastened the development in the rat of cataracts due to feeding high levels of galactose. In 4 rats fed a diet containing 10% of casein, Rezende and de Moura Campos ('42) observed bilateral cataracts. The parent rats had been fed the same deficient diet. According to Hall and Sydenstricker ('47) diets of this type are suboptimal with respect to several amino acids in addition to the more striking deficiency in methionine and cystine. Patch ('34) had previously described the occurrence of cataracts in the larva of *Amblystoma tigrinum* fed a highly purified milk-powder-casein diet. These could be prevented by adding cystine to the diet. Later, Patch ('43) observed cataracts in larva fed other proteins. These could not be prevented by supplementing the diet with cystine. Recently Ferraro and coworkers ('47) observed an early lens change consisting of vacuolation of the cortex, in rats fed a valine-deficient diet.

During an investigation of ocular changes in various deficiency states, we observed lenticular opacities in rats deficient in phenylalanine and in histidine. These observations led to the study reported here.

EXPERIMENTAL METHODS

Rats of a Wistar strain were used in this study. A description of the diets fed and the details of treatment of each group of rats are given in other sections of this paper. During the period of the experiment, biomicroscopic examinations of the eyes were made 3 or 4 times a week. In order to facilitate examination of the lens, usually the pupil of the right eye was dilated with a 0.5% solution of atropine sulfate

in 0.9% saline. Most of the deficient rats were allowed to die of the deficiencies. A few were changed to the control diet in an attempt to obtain regression of the lenticular changes. Also a few rats with some degree of lenticular changes were sacrificed in order that the lenses might be photographed.

On death or after killing the animal, the lenses were dissected out, immersed in saline and examined with a dissecting microscope. With the lenses immersed in saline and illuminated by 2 photo-flood lamps, photographs at a magnification of approximately $1\times$ were made on 35 mm panchromatic film. Usually a normal lens from a rat of approximately the same age was included for comparison.

All rats were kept in individual cages. Further details of care of animals and techniques are given in an earlier paper (Bowles and associates, '46).

RESULTS

Cataracts in phenylalanine deficiency

Since in the study of corneal changes in phenylalanine deficiency (Sydenstricker and co-workers, '47a), 1 of 11 rats was observed to develop a cataract, 25 more rats with 8 control animals from the same litters were placed, when 25 or 26 days of age, on the phenylalanine-deficient diet² described in that paper.

Definite lenticular changes were observed in all but 2 of the 25 rats on the phenylalanine-deficient diet in from 17 to 33 days (mean, 23 days). These changes consisted of a progressive lessening of the transparency of the lenses until

² The phenylalanine-deficient diet consisted of an amino acid mixture which supplied approximately 3 times the minimum requirement of each of the indispensable amino acids (except phenylalanine) in utilizable form, and the following: salt mixture 4 gm, cod liver oil 2 gm, cottonseed oil 3 gm, choline chloride 0.2 gm, riboflavin 1.6 mg, thiamine hydrochloride 0.4 mg, calcium pantothenate 2 mg, pyridoxine hydrochloride 0.4 mg and sufficient sucrose to make 100 gm. By appropriate modification of the amino acid content of this formula, histidine, threonine, leucine, isoleucine and protein-deficient diets were prepared.

at the time of death, after 20–35 days on the diet, various degrees of opacity were observed. The earliest change was a slight haziness of the lens substance. Next, the lens star became more readily visible. This was followed by a separation of the most superficial fibers, and sometimes the epithelial layer and possibly the superficial fibers appeared rough and granular. Frequently superficial, irregular, glistening areas were present when the lens was first removed, which disappeared after the lens had been in saline a few minutes. No changes in the lens capsule were observed. Later, after the appearance of a refractile line between nucleus and cortex, there developed in the nucleus a relatively dense central opacity. Figures 1, 2 and 4 show successive degrees of the superficial lens changes and the appearance of the refractile line. Figure 3 shows an early stage in the development of the central opacity.

The most extreme degrees of lenticular change in the rats deficient in phenylalanine could be observed satisfactorily only after death of the animal and removal of the lens. In the advanced stages of the deficiency disease the cornea became sufficiently opaque (Sydenstricker and co-workers, '47a) to make obscure the exact degree of lenticular change at the time when some of these rats were placed on the control diet in an attempt to produce reversal of the lenticular changes. However, before observation of the lenses with the biomicroscope became unsatisfactory in these rats and in those which died at this stage, superficial changes had developed to a moderate or extreme degree and the formation of a nuclear cataract had begun.

Five rats which had developed cataracts due to phenylalanine deficiency were changed to the control diet in an attempt to induce regression of the lenticular changes. This resulted in a complete reversal of the changes in the lenses except that the refractile line separating the superficial and deep portions of the lenses persisted. The corneal changes appeared to be completely reversed upon biomicroscopic examination. However, upon microscopic examination of the

dissected corneas, it could be seen that empty capillaries were still present.

Cataracts in histidine deficiency

The lenticular changes resulting from histidine deficiency were followed in 21 of our Wistar strain rats. Thirteen of these were the rats used by Sydenstricker and associates ('47a) in a study of the corneal changes in histidine deficiency. For 15 of the rats on the histidine-deficient diet and 8 rats fed a control ration, the details of the diets,³ care of the animals, and the techniques used were as described in the earlier paper. The remaining 6 histidine-deficient rats and 3 control animals from 2 litters were fed diets in which a histidine-deficient casein hydrolysate⁴ was used. The deficient diet consisted of histidine-deficient casein hydrolysate 14.5 gm, DL-tryptophane 0.4 gm, DL-methionine 0.3 gm, cod liver oil 2 gm, cottonseed oil 3 gm, salt mixture⁵ 4 gm, choline chloride 0.2 gm, riboflavin 1.6 mg, thiamine hydrochloride 0.4 mg, pyridoxine hydrochloride 0.4 mg, calcium pantothenate 2 mg, and a sufficient amount of sucrose to make 100 gm. The control diet was identical except that it contained 0.5 gm of L-histidine monohydrochloride in place of that amount of sucrose.

In histidine deficiency the early clouding of the superficial lens substance (fig. 5) was followed by an apparent granular disintegration of the more superficial fibers (fig. 6). This obscured any widening of the sutures that may have occurred. The development of opacity in the deeper lens substance was usually preceded by the appearance of a refractile line separating the superficial and deep portions of the lens (figs. 5 and 8). The final central opacity frequently was first ob-

³ See footnote 2, p. 279.

⁴ The histidine-deficient casein hydrolysate was prepared as described by Conrad and Berg ('37). The diets in which it was used were adaptations of those used by Conrad and Berg.

⁵ The salt mixture was that used by McKibben, Madden, Black and Elvehjem ('39).

served deep in the center of the lens (fig. 8), later spreading peripherally to the refractile line (fig. 9). Less often it was first seen just inside the refractile line (fig. 7) with subsequent extension deeper to the center of the lens. The resultant nuclear cataract was of variable size but usually of extreme density. Under the conditions of our experiment the lenticular opacities from histidine deficiency usually were of greater density than those which we observed to result from deficiency of phenylalanine, tryptophane or riboflavin. This was probably due to the fact that most of the histidine-deficient rats lived long enough for the cataracts to be fully developed, while the rats deficient in phenylalanine, or tryptophane or riboflavin usually died of the deficiency shortly after the lenticular opacities had begun to appear.

The lenticular changes due to histidine deficiency appeared after the rats had been fed the histidine-deficient diet from 10 to 34 days (mean 25 days); all of the rats on this diet, which lived long enough, developed cataracts.

Excepting 1 rat which died shortly after being placed on the deficient diet, the histidine-deficient rats lived from 34 to 61 days (mean 42 days) after being placed on the diet.

There were no significant differences between the lenticular changes observed in the rats on the 2 histidine-deficient diets. The control rats for the histidine-deficient casein hydrolysate diet developed no lenticular changes, though they did show some degree of corneal vascularization. This would indicate that this diet was not adequate in every respect.

When 3 rats with cataracts due to histidine deficiency were put on the control diet, the degree of reversal of lenticular changes depended on how far they had progressed. In any case the more superficial changes regressed. If a refractile line had formed separating the deeper and outer portion of the lens, this would persist after other lenticular changes had disappeared (fig. 11). This corresponds to what was observed in the regression of cataracts due to phenylalanine deficiency. When 2 deficient rats were returned to the

control diet after the central opacity had begun to form, although a reversal of other changes began immediately, the formation of the dense central cataract went to completion (figs. 10 and 12). Once formed, the dense central cataract did not regress.

Cataracts in tryptophane deficiency

In order to make comparable studies of cataracts due to tryptophane deficiency, 23 rats 21 to 27 days of age were placed on a tryptophane-deficient diet. Ten rats from the same litters were placed on a control diet. The tryptophane-deficient diet consisted of zein⁶ 30 gm, L-histidine monohydrochloride 0.3 gm, L-lysine monohydrochloride 1.35 gm, DL-valine 0.8 gm, DL-threonine 0.3 gm, DL-methionine 0.3 gm, salt mixture⁷ 4 gm, cottonseed oil 3 gm, cod liver oil 2 gm, choline chloride 0.2 gm, calcium pantothenate 2 mg, thiamine hydrochloride 0.4 mg, riboflavin 1.6 mg, pyridoxine hydrochloride 0.4 mg, and a sufficient amount of sucrose to make 100 gm. The control diet was identical with this except that it contained 0.4 gm of DL-tryptophane in place of a like amount of sucrose.

With tryptophane deficiency the initial changes in the lenses were observed to consist of an apparent loosening and slight separation of superficial fibers, both anteriorly and posteriorly with widening of suture lines, and sometimes a radiating opacity of the superficial fibers, both anteriorly and posteriorly with widening of suture lines, and sometimes a radiating opacity of the superficial fibers around the lens star. Later a concentric, refractile line appeared similar to what was observed in lenses of rats deficient in phenylalanine and histidine. Within the central portion there appeared reduction in transparency varying from slight, diffuse haziness to dense, white opacity. Frequently the opacity of this cen-

⁶ Obtained from the Corn Products Refining Co., New York, N. Y.

⁷ See footnote 5, p. 281.

tral portion was more dense peripherally, giving a ring-shaped appearance to the cataract. The peripheral portion of the lens, outside the refractile line, showed changes varying from slight haziness to moderate, fine diffuse opacity. The lenticular changes which were observed in tryptophane deficiency are illustrated in figures 13, 14 and 15. Our observations correspond essentially to those made by Totter and Day ('42), Albanese and Buschke ('42) and other investigators.

*Lenticular changes in deficiencies of protein
and of amino acids*

The lenses from several rats used in the investigation of another problem were dissected and examined for abnormalities. Eight of the rats were on a protein-free diet^s as used by Hall and co-workers ('46), 7 on a threonine-deficient diet,^s 1 on a leucine-deficient diet^s and 9 on an isoleucine-deficient diet^s as described by Sydenstricker and associates ('47a).

In all of the 8 rats which had been on a protein-free diet some forms of early lenticular changes were observed similar to those described above for phenylalanine deficiency. These consisted of haziness, visible separation of superficial fibers and widening of the sutures in varying degree.

The lenses from 4 of the 7 rats on the threonine-deficient diet and from the rat on the leucine-deficient diet showed similar changes.

The dissected lenses from the 9 rats on the isoleucine-deficient diet appeared to be normal except for a questionable widening of the suture line in the eyes of 1 animal.

These observations and the fact that a definite haziness of the lenses was seen with the biomicroscope in these and other amino acid deficiencies has led us to conclude that lenticular changes of some degree may result in the rat from deficien-

^s See footnote 2, p. 279.

cies of protein or of any of the indispensable amino acids except arginine.

Cataracts in riboflavin deficiency

Twelve rats from 2 litters were placed, at weaning, on a riboflavin-deficient diet which contained vitamin-free casein ⁹ 20 gm, salt mixture ¹⁰ 4 gm, U.S.P. cod liver oil 2 gm, cottonseed oil 3 gm, choline chloride 0.2 gm, pyridoxine hydrochloride 0.4 mg, calcium pantothenate 2 mg, thiamine hydrochloride 0.4 mg and sufficient sucrose to make 100 gm. In addition to the 12 rats used here, at various times we have made biomicroscopic examination of the lenticular changes occurring in other riboflavin-deficient rats which were available for such study. While there were no litter-mate controls for the 12 rats on the riboflavin-deficient diet, we have had at various times 71 rats which were fed for different periods this same diet but with added riboflavin. None of the 71 rats developed lenticular changes which could be observed with the biomicroscope.

In riboflavin deficiency the initial lenticular changes observed were a loosening and separation of superficial fibers and slight opening of sutures. Frequently, immediately after removal of the lens, there were seen in the loosened superficial fibers irregular areas of glistening, gray opacity which disappeared completely after the lens had been in saline a few minutes. This is similar to what was seen in phenylalanine-deficiency. The more advanced changes observed consisted of diffuse granular opacity in the superficial lens substance, the appearance of a refractile line between cortex and nucleus and development of a dense, white, spherical nuclear cataract. The lenticular changes observed in riboflavin-deficiency are illustrated in figures 17 and 18.

Our observations are similar to those made by Day, Langston and O'Brien ('31) with regard to this type of cataract.

⁹ Labco brand.

¹⁰ See footnote 5, p. 281.

Spontaneous cataracts

Out of some 1200 rats from our colony of Wistar strain animals, the eyes of which have been examined with the biomicroscope, in only 2 litters (8 and 5 rats, respectively) have spontaneous cataracts been observed. In the first litter cataracts were observed to appear almost simultaneously in 5 of the 8 rats when they were 104 days of age. The other 3 rats from the litter had previously been used in another experiment and killed. The cataracts were in the form of dense, cottonlike, nuclear opacities in otherwise apparently normal lenses. The size and density of the cataracts increased for 7 to 10 days at which time they varied from the size illustrated in figure 16 to approximately one-fourth of the diameter of the lens. Three of the animals were sacrificed for study and photographing of the lenses. The eyes of the remaining 2 rats were examined at periodic intervals for 51 and 112 days longer but no further lenticular changes took place.

The second litter of rats with spontaneous cataracts was the result of breeding 2 rats from a litter which consisted of runts. Of the 5 offspring, 4 were nearly normal in size, and the fifth, a runt. All 5 had cataracts when the eyes were first examined at 28 days of age. All except 1 had bilateral cataracts which were bilaterally quite unsymmetrical. Six of these cataracts, when first observed, were central nuclear cataracts of varying sizes which increased until the smallest were about the size of cataracts shown in figure 16 and the largest filled two-thirds of the diameter of the lens. Of 2 cataracts which were markedly eccentric when first observed, 1 spread across the lens to form a roughly spherical central cataract. The other spread around the circumference of the nucleus to form a ring-shaped cataract which eventually spread to the center. One of the cataracts which was ring-shaped when first observed, after forming radial white projections to the center of the lens, developed into a dense, white, central cataract. One eye of 1 rat appeared normal. The other eye of this rat contained the smallest and least

dense of the cataracts seen in this litter. Except for the nuclear opacities, the lenses all appeared to be normal. The cataracts developed rapidly for 7 to 14 days from the time they were first observed and then increased slightly in size and density during the next 6 weeks. Hereditary cataracts in the rat have been observed by Bourne and Grüneberg ('39) and by Lambert and Sciuchetti ('35).

Cataracts in a rat on a high tyrosine diet

Out of 73 rats of varying ages which were fed a 20% casein diet containing 10% additional L-tyrosine, 1 young adult rat developed large, dense, nuclear cataracts after 6½ months on this diet (fig. 27). None of the other rats, all of which had been on the diet for shorter periods of time, developed cataracts. Various organic compounds of the aromatic series, of which tyrosine is a member, are known to produce cataracts.

DISCUSSION

In general, there are marked similarities between the degenerative changes in the lens resulting from deficiencies of protein, amino acids or of riboflavin. These changes are: general haziness of the lens, separation of the superficial fibers, widening of the sutures, diffuse granular opacities in the cortex, the appearance of a refractile line separating cortex and nucleus, and dense nuclear opacity of variable extent.

The spontaneous cataracts seen in the 2 litters were similar in appearance but more irregular in outline and were not associated with the superficial changes seen in the nutritional deficiencies.

In general we have observed the development of corneal changes in protein, amino acid and riboflavin deficiencies to be bilaterally more uniform than were the lenticular changes. In table 1 a summary is given and a comparison is made of the corneal and lenticular changes in protein, amino acid and riboflavin deficiencies. This table shows for these defi-

TABLE 1

Summary of corneal and lenticular changes observed in young rats fed diets deficient in protein, amino acids or riboflavin.¹

DEFICIENCY	CORNEA			LENS				
	Vasculari- zation	Opacity	Superficial ulceration	Superficial changes			Deep changes	
				Haziness	Separation of fibers	Widening of sutures	Line separating cortex and nucleus	Nuclear opacity
Protein	+	+	+	+	+			
Methionine	+	+		+				
Phenylalanine	+	+	+	+	+	+	+	+
Leucine	+	+		+	+			
Isoleucine	+	+		+				
Arginine	+	+						
Histidine	+	+		+	+	+	+	+
Threonine	+	+		+	+			
Valine	+	+		+				
Lysine	+	+		+				
Tryptophane	+	+	+	+	+	+	+	+
Riboflavin	+	+		+	+	+	+	+

¹ The corneal changes summarized in this table were described by Hall et al. ('46), Berg and co-workers ('47), Sydenstricker and associates ('47a) and Bowles et al. ('46). The above estimates of the degree of corneal changes in specific deficiencies take into account the extent and severity of the particular change, the rapidity of its development, and the length of time the rat lived after the change first appeared.

ciencies a similarity between the extent and severity of the changes in the cornea and those in the lens, but with the exception that in histidine deficiency the lenticular changes are more extreme.

It is of interest to note that Mitchell ('36) found Wistar strain rats, such as were used in this study, to be more resistant than some other strains to the development of cataracts due to the feeding of high levels of lactose or galactose.

No lenticular changes were observed in any of the control rats used in this study.

The weight changes of the rats on the experimental diets in this study were essentially the same as those reported by Sydenstricker et al. ('47a) and Bowles and co-workers ('46) where similar diets were used.

Day, Darby and Cosgrove ('38) were able by the administration of riboflavin to arrest the development of the dense central cataracts in some riboflavin-deficient rats. In the present study the number of rats deficient in phenylalanine and in histidine in which an attempt was made to produce a regression of lenticular changes was too small to determine conclusively whether such an arrest was possible.

The routine use of atropine sulfate to dilate the pupils of the right eyes of the rats used in this study resulted in a more extreme keratitis of the cornea than in the left eyes of these rats where atropine was used only occasionally, or than in the eyes of other rats on the same diets where biomicroscopic examinations were made as frequently but where the pupils were not dilated. The use of atropine had no effect on the development of the cataract.

SUMMARY

The lenticular changes observed in rats deficient in phenylalanine, histidine, tryptophane or riboflavin were: general haziness, separation of the superficial fibers, widening of the sutures, diffuse and granular opacities in the cortex, granular degeneration of the superficial cortex, the appearance of a refractile line separating cortex and nucleus, and

dense nuclear opacity of variable extent. The characteristic changes in each deficiency are described and illustrated. Lenticular changes of some degree were observed in deficiency of protein and of each of the indispensable amino acids except arginine. When rats deficient in phenylalanine or histidine were returned to the control diet, a reversal of superficial changes resulted, though the dense nuclear opacities were found to be irreversible.

The spontaneous cataracts observed in 2 litters of rats, and cataracts in a rat on a high tyrosine diet are also described.

ACKNOWLEDGMENTS

This study was aided by grants from the John and Mary R. Markle Foundation and from the Division of Grants, National Institute of Health, U. S. Public Health Service. Some of the amino acids used in this study were donated by Winthrop Chemical Company. We thank Nathaniel Thornton, II, for assistance, and Elizabeth Thompson for care of the animals.

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PLATE 1

EXPLANATION OF FIGURES

(All $7\times$, reduced approximately three-eighths)

Photographs of lenses dissected from rats which had been on normal diet and on deficient diets. In each are the 2 lenses from 1 rat with a normal lens in the center except in 9 where a normal lens is not included and in 10 where the normal lens is at the left.

1 through 4 Phenylalanine-deficient diet for 46, 36, 30 and 31 days, respectively.

5 through 9 Histidine-deficient diet for 34, 45, 37, 34 and 62 days, respectively.

10, 11 and 12 Histidine-deficient diet for 31, 58 and 40 days and control diet for 21, 27 and 22 days, respectively.

CATARACTS IN THE RAT

HALL, BOWLES, SYDENSTRICKER AND SCHMIDT

PLATE 1

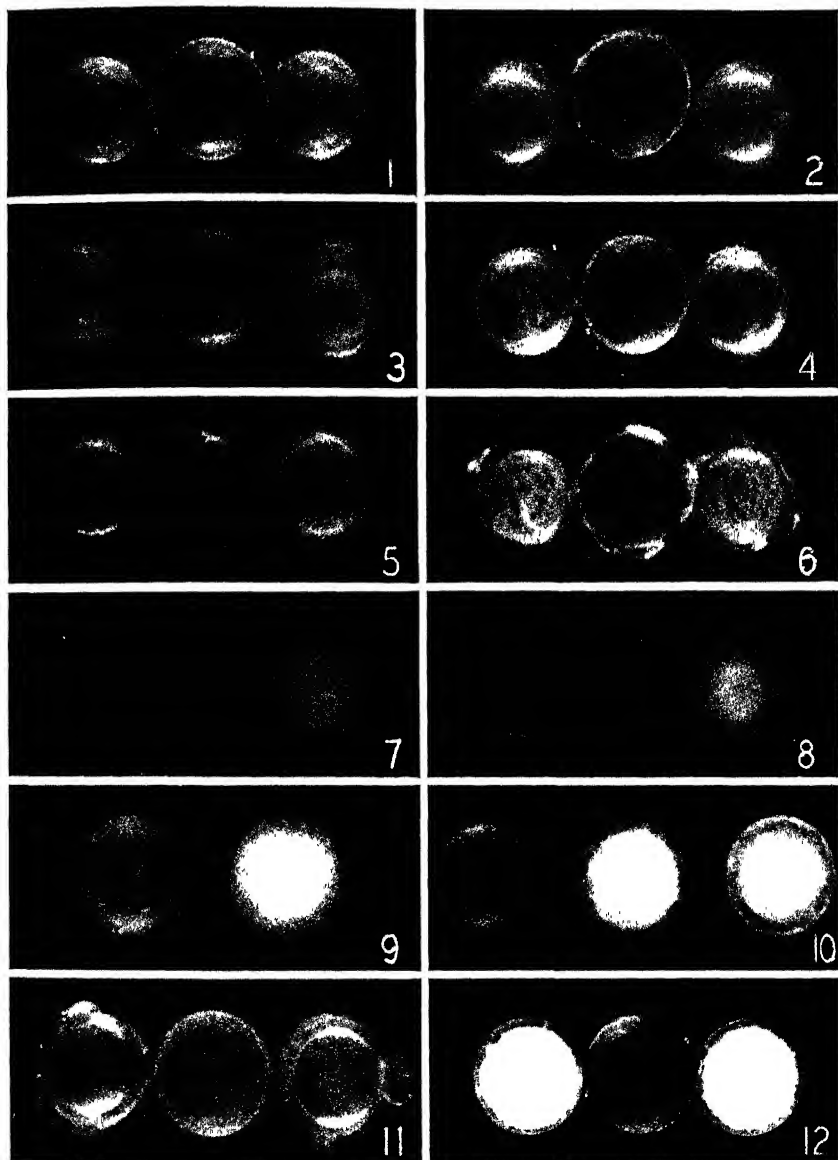


PLATE 2

EXPLANATION OF FIGURES

(All 7 X, reduced approximately three eighths)

13 through 18 Photographs of lenses dissected from rats which had been on normal diet and on deficient diets. In each are the 2 lenses from 1 rat with a normal lens in the center except in 13 where a normal lens is not included.

13, 14 and 15 Tryptophane-deficient diet for 23, 62 and 51 days, respectively.

16 Spontaneous cataracts, observed in the rat when 104 days of age. Rat killed and photographed 7 days later.

17 and 18 Riboflavin-deficient diet for 119 and 104 days, respectively.

19 through 26 Photographs of eyes of rats which had been on normal diet and on deficient diets. The 2 highlights in each are reflections of the photoflood lamps and should be disregarded.

19 Histidine-deficient diet for 45 days. The lens from this eye is in 6.

20 Normal diet.

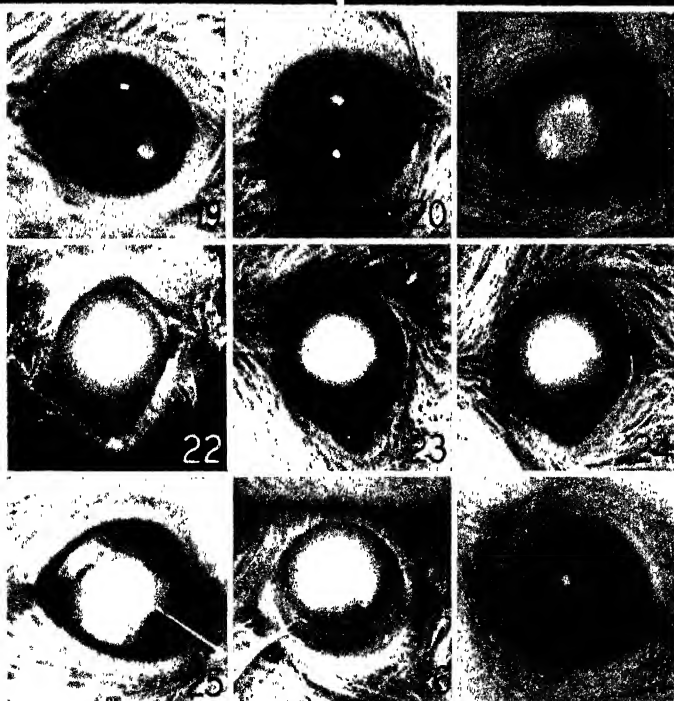
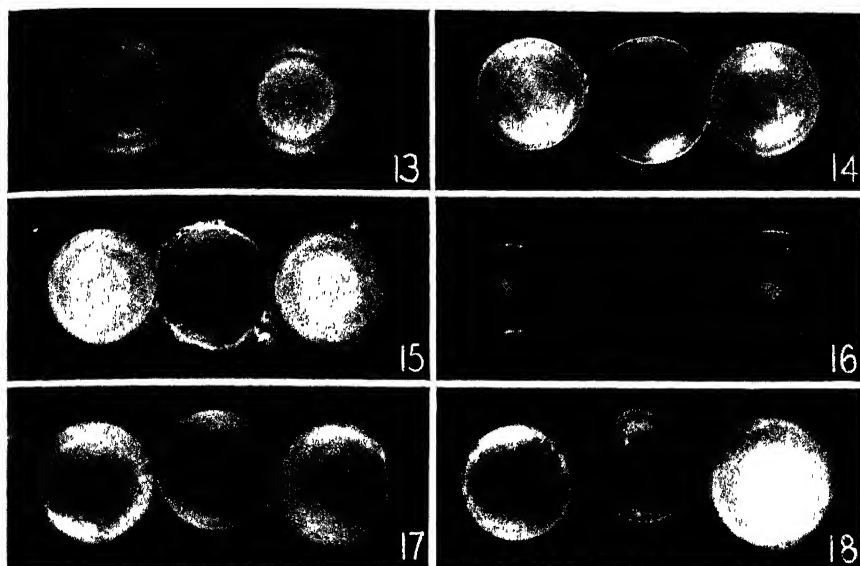
21 and 22 Histidine-deficient diet for 34 and 62 days. The lenses from these eyes are on the right in 8 and 9.

23 and 24 Histidine-deficient diet 31 days, and control diet 21 days. The lenses from these eyes are in 10.

25 Histidine-deficient diet 40 days and control diet 22 days. The lens from this eye is in 12.

26 Tryptophane-deficient diet for 23 days. The lens from this eye is on the right in 13.

27 Eye of a rat on high-tyrosine diet for 196 days. (The vessels of cornea and iris show injection with India ink. The photograph of the eye of a normal control rat which had been similarly injected was indistinguishable from the photograph of the normal uninjected eye shown in figure 20).



THE RELATION OF PYRIDOXINE TO THE GROWTH OF CHICKS FED RATIONS CONTAINING LINSEED OIL MEAL

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TWO FIGURES

(Received for publication March 24, 1948)

Numerous workers have demonstrated that linseed oil meal is detrimental to growing chickens when it is fed at levels of 4.5% or more of the ration (Bethke et al., '28; Ackerson et al., '38; Sherwood and Couch, '40; Slinger et al., '43; Hammond, '44; Heuser and Norris, '44; Heuser et al., '46). However, the nutritional value of linseed oil meal for promoting growth in chicks may be improved by treating the meal with water (Kratzer, '46; McGinnis and Polis, '46; Kratzer, '47; MacGregor and McGinnis, '48). It has also been shown that improvement approximately equal to that observed following the water treatment can be produced by the addition of a mixture of synthetic vitamins to a ration containing untreated linseed oil meal (Kratzer and Williams, '48). The problem has been studied further in an attempt to determine the relationship of the addition of various vitamins to the improvement in growth and to elucidate the mechanism of the changes brought about by the water treatment.

PROCEDURE AND RESULTS

Methods

Single Comb White Leghorn chicks were fed a standard starting ration for a period of about 10 to 14 days, after which they were divided into groups and were fed the experimental rations. The chicks were kept in electrically heated batteries with raised wire floors and were supplied with feed and water ad libitum. The experiments were continued for 18 to 20 days.

The experimental rations contained the following: crude casein, 5.0 gm; dried whey, 10.0 gm; alfalfa meal, 10.0 gm; steamed bone meal, 1.0 gm; cottonseed oil, 5.0 gm; ground limestone, 2.0 gm; salt, 1.0 gm; manganese sulfate, 0.0125 gm; fish oil (1000 A, 400 D), 0.125 gm; ground wheat, 20.0 gm; ground barley, 16.0 gm and either untreated or water-treated linseed oil meal 30.0 gm. The linseed oil meal used¹ was an experimentally produced, solvent-processed sample differing from the old-process linseed oil meal used in previous work. The vegetable oil was found essential to prevent the feed from sticking to the beaks of the chicks. The vitamin mixture which was fed to certain groups supplied per kilogram of diet: thiamine hydrochloride 20 mg; riboflavin 25 mg; pyridoxine hydrochloride 35 mg; calcium pantothenate 110 mg; nicotinic acid 200 mg; pteroylglutamic acid² 10 mg; inositol 4 gm and choline 4 gm. In the second experiment biotin³ was included in the mixture at the level of 0.2 mg per kilogram. When additions of single vitamins were made, they were at the above levels. The water treatment of the linseed oil meal was carried out by mixing the meal with 3 times its

¹ Kindly donated by Archer-Daniel-Midland Company, Minneapolis, Minn., through the courtesy of Dr. J. W. Hayward.

² Kindly donated by Lederle Laboratories, Inc., Pearl River, New York, through the courtesy of Dr. T. H. Jukes.

³ Kindly donated by Merck and Company, Rahway, New Jersey, through the courtesy of Dr. D. F. Green.

weight of water and allowing it to stand for 1 day at room temperature. The material was then dried at approximately 65°C. and ground in a burr mill.

*A. Qualitative study of vitamin supplementation
of linseed oil meal*

In the first experiment the effect of the addition of various vitamins to the ration containing untreated linseed oil meal

TABLE 1

*The effect upon the growth of chicks of water treated linseed oil meal
and/or adding vitamins*

SUPPLEMENT	AVE. GAIN IN WEIGHT (GM)	
	Trial A	Trial B
<i>Experiment 1 — Trial A, 18 days; B, 30 days</i>		
Linseed oil meal	34	42
Linseed oil meal + vitamin mix	118	198
Linseed oil meal + pyridoxine	86	181
Linseed oil meal + inositol	53	159
Linseed oil meal + choline	70	
Linseed oil meal + pyridoxine + inositol	92	
Linseed oil meal + pyridoxine + inositol + choline	105	
Water-treated linseed oil meal	78	171
Water-treated linseed oil meal + vitamin mix	128	
<i>Experiment 2 — 19 days</i>		
Linseed oil meal	97	
Linseed oil meal + pyridoxine	166	
Linseed oil meal + vitamin mix	169	
Linseed oil meal + inositol	105	
Water-treated linseed oil meal	180	
Water-treated linseed oil meal + pyridoxine	188	
Stock mash	191	

was tested. The vitamin additions which were made and the results obtained are shown in table 1. The addition of the vitamin mixture caused the greatest increase in growth. Pyridoxine alone caused a large increase in growth, but in both trials this was not equal to the growth produced by the

vitamin mix. Inositol caused a slight increase in growth when it was added to the ration containing no other vitamin supplements, but caused no response in 2 trials when it was added to rations supplemented with pyridoxine. Choline caused an increase in growth when it was added to the ration either alone or in combination with pyridoxine and inositol. The water treatment of linseed oil meal caused a growth increase approximately equal to that caused by the addition of pyridoxine, but it was not as great as the increase caused by the vitamin mixture. These data were interpreted to indicate that the water treatment increased growth by eliminating the need for added pyridoxine, but the water-treated linseed oil meal ration was still suboptimal with respect to choline. This deficiency of choline may have resulted from the solvent extraction process while the old-process linseed oil meal used in earlier work was adequate in choline.

To test the supposition that the water treatment resulted in an improvement of linseed oil meal for chicks by virtue of eliminating the necessity for supplying pyridoxine to the ration, a second experiment was conducted in which the basal ration contained additions of the B-complex vitamins. The basal ration was similar to that originally described with the exception of the following additions: choline 0.1 gm; inositol 0.1 gm; thiamine hydrochloride 0.1 mg; riboflavin 0.2 mg; calcium pantothenate 0.6 mg; nicotinic acid 0.9 mg; biotin 0.005 mg and pteroylglutamic acid 0.05 mg per 100 gm. The groups included in the trial are shown in table 1. Marked improvement in growth occurred when pyridoxine was added to the untreated linseed oil meal; none occurred when it was added to the water-treated meal. There was no improvement beyond that caused by the addition of pyridoxine when the vitamin mixture was added. The addition of inositol caused no appreciable response; however, the basal ration in this trial contained inositol. The data indicate that the major part of the response, if not all, resulting from the water treatment of linseed oil meal was due to eliminating the need for added pyridoxine.

*B. Quantitative relationship of linseed oil meal
and pyridoxine*

Since it was clearly indicated that pyridoxine alleviated the growth inhibiting properties of linseed oil meal, it seemed desirable to determine the amount of pyridoxine required to prevent the detrimental effects. A basal ration was used which contained 30% solvent-processed linseed oil meal and was similar to that used in the second experiment of part A.

Varying amounts of pyridoxine hydrochloride were added to this ration. Ten chicks per group were continued on these

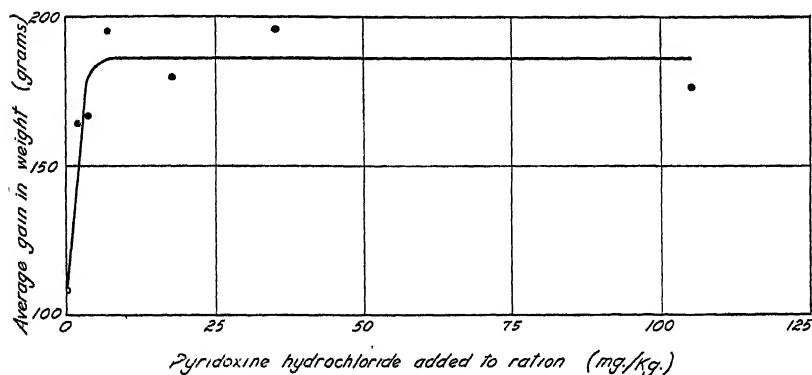


Fig. 1 Relation of the level of pyridoxine hydrochloride added to a ration containing linseed oil meal to the growth of chicks.

rations 20 days. The results are shown in figure 1. Maximum growth was obtained with the addition of 7 mg per kilogram, while 1.75 and 3.5 mg per kilogram produced growth intermediate between the 7 mg level and the group receiving only the basal ration. This was assayed for pyridoxine by the use of *Neurospora sitophila*⁴ (Stokes, '47) and was found to contain 12.5 mg per kilogram.

In other groups of the same experiment no supplementary pyridoxine was added to the ration but the level of linseed oil meal was varied from 0 to 30%. The crude protein content

⁴ Kindly donated by Dr. G. W. Beadle, California Institute of Technology, Pasadena.

of the rations was maintained constant by the addition of soybean oil meal and ground wheat as the linseed oil meal was reduced. The growth data are presented in figure 2. A reduction in growth was not apparent until the level of linseed oil meal in the ration exceeded 10%, but the reduction was very rapid as the linseed oil meal was increased beyond this level. The curve is admittedly subject to some error due to

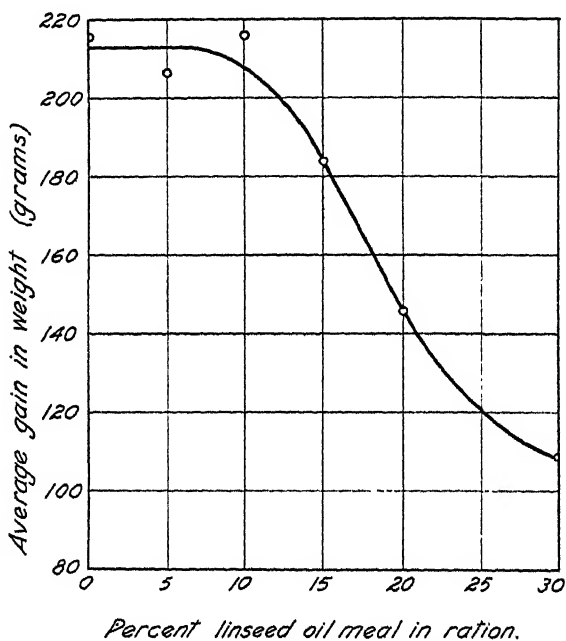


Fig. 2 Relation of the level of linseed oil meal in the ration to the growth of chicks.

variations in the amino acid content of the rations as the preparations of linseed, soybean and wheat proteins varied, but it is felt that the greatest differences were due to the specific growth depressing action of linseed meal. This is substantiated by the fact that groups receiving 30% linseed oil meal and supplemented with adequate pyridoxine grew nearly as rapidly as those receiving the low levels of linseed oil meal.

DISCUSSION

The data indicate that linseed oil meal depressed growth of chicks by rendering the ration deficient in pyridoxine. However, the total pyridoxine in the ration (12.5 mg/kg) was many times the level of 2.0 to 3.0 mg per kilogram which has been reported to be required by chicks for normal growth in rations which contain no linseed oil meal (Briggs et al., '42; Kratzer et al., '47). The principal effect of the water treatment of linseed oil meal was to reduce the requirement for pyridoxine. The linseed oil meal itself contained approximately 18 mg of pyridoxine per kilogram. Thus, the amount supplied to the ration by 30% linseed oil meal was approximately 5.4 mg per kilogram. It can therefore be assumed that the non-linseed components of the ration supplied over 7 mg of pyridoxine to each kilogram of the ration.

The mechanism by which the pyridoxine deficiency is created is not apparent. There are at least 2 ways by which this could occur. First, if linseed contained a factor capable of combining with pyridoxine to form a complex which would render pyridoxine unavailable, a deficiency would result. This would be analogous to the action of avidin in rendering biotin unavailable and thereby creating a deficiency of biotin (Eakin et al., '40). In such a situation the water treatment would have to destroy the pyridoxine-combining compound so that it could not form a complex.

A second theory for the apparent pyridoxine deficiency involves the presence in linseed oil meal of a compound which is similar enough to pyridoxine to substitute for it in biologically important complexes, but different enough so that it does not possess biological activity, thus making it a competitive inhibitor. Many such analogues have been recognized (Woolley, '47) but for the most part they have not been found in natural products. In such a theory the water treatment would be expected to destroy the competitive inhibitor or inactive analogue so it could not compete with pyridoxine. The addition of pyridoxine would replace that rendered inactive by the

supposed competitive inhibitor and provide the biologically active pyridoxine needed.

The fact that previous workers observed growth reduction at levels of linseed oil meal of 4.5% and higher while in the present work 10% seemed to be the critical level, probably reflects differences in the pyridoxine content of the basal rations used rather than differences in response. It is probable that our ration contained more pyridoxine than the former rations and it required a higher level of linseed to create an apparent pyridoxine deficiency.

The exact mechanism for the creation of an apparent pyridoxine deficiency in chicks by feeding linseed oil meal and the processes by which linseed oil meal can be altered to prevent this action by water treatment must await further experimentation.

SUMMARY

When linseed oil meal was fed to chicks at 30% of the ration an apparent pyridoxine deficiency resulted, although the ration contained many times the amount of pyridoxine normally required by chicks. The deficiency was prevented by adding synthetic pyridoxine or by treating the linseed oil meal with water.

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ADDENDUM

Subsequent determinations of the pyridoxine content of linseed oil meal by the *Neurospora sitophila* method have shown considerable variations. Chemical analyses made by Mr. W. R. Flach of the Eastern States Farmer's Exchange, Buffalo, New York, indicated that both untreated and water-treated linseed oil meal contained approximately 6 mg of pyridoxine per kg.

There was no increase in pyridoxine resulting from the water treatment, and the amount present in the ration as found by either method was many times the required level.

THE RELATION BETWEEN URINARY EXCRETION AND TISSUE CONCENTRATIONS OF THIAMINE IN RATS

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ONE FIGURE

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Although the thiamine concentration of tissues has been studied by a number of workers (Westenbrink, '32; Brodie and McLeod, '35; Leong, '37; Schultz et al., '39; Ferrebee et al., '42; Mitchell and Isbell, '42; Sure and Ford, '42; Sarett and Perlzweig, '43; Schweigert et al., '43) under varying conditions, little attempt has been made to correlate tissue levels with urinary excretion. Since clinical evaluation of deficiency situations is in large part dependent upon urinary excretion data, it is of obvious importance to interpret such data in terms of what is going on in the tissues. The present experiments were undertaken to fill this gap.

EXPERIMENTAL

Plan

The general plan was to deplete a group of rats with respect to thiamine, sacrificing representatives of the group at intervals for tissue analysis and following the total urinary

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output of thiamine during the depletion process. Particular interest is attached to the state of the tissues when the "point of minimum excretion" in the urine was attained, since it has been postulated (Holt and Najjar, '43) that excretion above this level corresponds to a surplus above physiological needs and depletion beyond this point to potential physiological deficiency.

Animals

The animals employed were adult males from a colony of mixed albino and hooded Norwegian rats. They were kept in individual metabolism cages to permit collection of urine.

Diet

After a period on the standard McCollum diet the animals were placed on a thiamine deficient diet identical with that described in a previous publication (Najjar and Holt, '43) with the exception that sucrose was employed in place of dextrimaltose.

Analytical methods

Urine was preserved with acetic acid and analyzed for thiamine by a modification (Najjar and Ketron, '44) of the standard thiochrome procedure (Hennessy and Cerecedo, '39). Analyses of free and combined thiamine (cocarboxylase) were carried out on brain, heart, kidney, liver and muscle. For free thiamine a sample of fresh tissue was macerated and extracted with boiling 2% acetic acid for 20 minutes. It was then centrifuged and the supernatant fluid decanted for thiamine analysis. For total thiamine the acetic acid extract, prepared as described above, was treated with takadiastase, the pH adjusted to 4.5 with alkali and incubated at 45°C. for 1 hour with occasional shaking. This was followed by centrifugation at high speed, then by decanting and analysis of the supernatant fluid. The combined thiamine was determined by difference.

RESULTS

Two groups of rats were studied. The first group of 12 rats served essentially as an orientation experiment to determine how long it would take for the urinary excretion of thiamine to fall to the minimum point and at what intervals rats should be sacrificed for tissue analysis. It was found that from 6 to 16 days were required for the urine to reach the minimum level in individual animals; the group as a whole

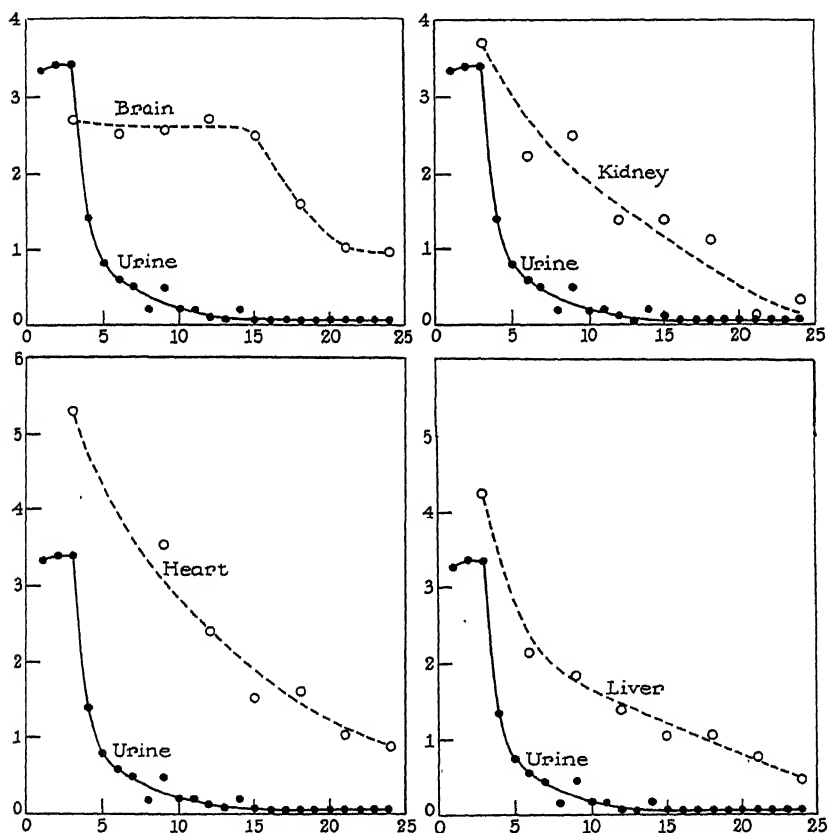


Fig. 1 Relation between thiamine of tissues and thiamine excretion in urine. Ordinates represent micrograms thiamine per gm dry tissue and micrograms per day of thiamine in urine. Abscissae represent days on thiamine deficient diet. The tissue figures represent averages of 4 animals sacrificed. The urine figures represent averages of surviving animals.

maintained a minimum excretion from the 17th day onward. Assays of tissues were made on animals sacrificed on the 9th and on the 31st day of the deficient diet. These assays were obviously not suitable for any correlation of tissue changes with the point of minimum excretion, but they were of interest in showing that virtually all of the tissue thiamine was combined, and that the combined thiamine of the brain (in contrast to other tissues) was maintained at least up to the 9th day although it fell thereafter.

A second experiment was then carried out with 32 rats, 4 of which were sacrificed for analysis every 3 days beginning on the 3rd day of the experimental period. Pooled urines of the surviving rats were assayed for thiamine each day. The results of this experiment are shown graphically in figure 1 where the total thiamine of the tissues and the urinary thiamine are shown through the period of developing deficiency. Separate analyses of free and combined thiamine of the tissues were carried out, but since the quantities of free thiamine were negligible and did not influence the picture, only the total thiamine figures are given.

It will be noted that the concentration of thiamine in the liver, kidney and heart falls steadily throughout the period of developing deficiency in a curve which shows no breaks and is of a logarithmic type. In sharp contrast is the behavior of the brain thiamine which is sustained at a constant level until the point of minimal urinary excretion is reached after which it falls sharply.

DISCUSSION

The data on tissue thiamine concentrations which we have reported above are in good agreement with those in the literature. It is clear that the thiamine concentration of most organs varies directly with the diet over a wide range of intakes, although there appears to be an upper limit (Schultz et al., '39) beyond which the tissue concentration cannot be increased. The tendency of the brain to maintain its thiamine concentration in the face of a reduced intake has also been

reported by others (Westenbrink, '32; Brodie and McLeod, '35). We have been able to find simultaneous determinations of tissue thiamine and urinary thiamine only in the work of Ferrebee et al. ('42), but these authors, although they compared normal animals with those on a deficient diet, did not study the progress of the deficiency changes in the tissues and urine or attempt a correlation between them.

It would appear from the data at hand that the brain differs from other tissues studied in not being able to store surplus thiamine. The sudden break in the concentration of brain thiamine when depletion reaches the point of minimum urinary excretion suggests the development of unphysiological changes in this tissue and is in keeping with the known susceptibility of the nervous system to thiamine deficiency. That the "point of minimum excretion" of thiamine in the urine is compatible with health and is not far above a level at which deficiency symptoms make their appearance is indicated by studies made by some of us of experimental thiamine deficiency in adult and adolescent humans (Najjar and Holt, '43; Holt, '44). Further studies made on infants (Holt et al., '46; Snyderman et al., to be published), although not carried to the point of deficiency symptoms, have shown that an intake which maintains the infant at the point of minimum excretion may be continued for long periods without evidences of clinical deficiency. Since the point of minimum excretion can be readily determined, it provides what appears to be a highly useful end point for determining thiamine requirements under various circumstances.

SUMMARY

1. The thiamine content of rat tissues has been studied under conditions of developing thiamine depletion and has been correlated with the excretion of thiamine in urine.

2. Unlike other tissues studied the brain maintains its thiamine concentration in the face of a deficit of thiamine for a considerable period, after which there occurs an abrupt fall in thiamine content.

3. The critical point at which the brain begins to lose its thiamine corresponds to the attainment of the minimum level of urinary thiamine excretion. This finding supports the view that the point of minimum urinary excretion is of physiological significance.

4. It is suggested that the point of minimum excretion in the urine is a highly useful criterion for measuring thiamine requirements under various conditions.

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VITAMIN STUDIES IN MIDDLE-AGED AND OLD INDIVIDUALS

I. THE VITAMIN A, TOTAL CAROTENE AND $\alpha + \beta$ CAROTENE CONCENTRATIONS IN PLASMA¹

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ONE FIGURE

(Received for publication February 24, 1948)

INTRODUCTION

In spite of the existence of an extensive literature dealing with the clinical aspects of vitamin A, data on the plasma concentration of this vitamin and of carotenes in middle-aged and old individuals are still scanty. The present investigation was undertaken with the purpose of supplying such information.

The study comprises analyses of plasma total carotenes, $\alpha + \beta$ carotenes and vitamin A in 155 individuals (71 men and 84 women) between the ages of 40 and 99. For comparison 47 younger individuals (22 men and 25 women) between 16 and 39 years were included in the investigation. The majority of the older subjects were inmates or patients in the St. Louis City Infirmiry and City Infirmiry Hospital, whereas the greater part of the younger individuals examined were private volunteers. The study was undertaken during the months July to September, 1947. During this period the diet offered to the patients in the 2 above-mentioned institutions

¹Funds and materials provided by Hoffmann La Roche, Inc.

was calculated to contain an average of 2,000 and 4,000 μg of vitamin A (vitamin A + carotene) daily, a content which is considered adequate for adult individuals. None of the patients examined was given supplementary vitamin preparations during the weeks preceding the blood analysis. The vitamin content of the diet of the private group of younger individuals was not evaluated.

METHODS

The technique used was a modification of those recommended by The Association of Vitamin Chemists (Methods of Vitamin Assay, '47), Willstaedt and With ('38) and With ('47). The carotenes and vitamin A were extracted from the saponified plasma sample by successive portions of ether. After evaporation of the ether, the residue was dissolved in petroleum ether. The total carotenes were determined by use of a Dubosque colorimeter, and the $\alpha + \beta$ carotenes after previous adsorption on an aluminum oxide column and subsequent elution with benzol-benzene (fluid chromatography). The vitamin A was estimated on aliquots of the petroleum ether sample by the Carr-Price reaction. The more detailed technique will be given below. It will be noted that the customary treatment of the combined ether extracts with anhydrous sodium sulfate was omitted in the authors' procedure. This omission was undertaken because it was found that several samples of sodium sulfate examined yielded substances of a marked yellow color, which, furthermore, gave a strong Carr-Price reaction, thus interfering with both the carotene and the vitamin A determinations. Only after the exclusion of this step of the analysis were reproducible results obtained with constancy. It appears likely that this precaution accounts for the somewhat low vitamin A plasma values reported in this investigation. In order to avoid turbidity in the Carr-Price color development due to traces of water in the sample, a few drops of acetic anhydride (as recommended by With, '47) were added to the chloroform

solution before the addition of the antimony trichloride reagent. This practically always resulted in samples completely free from turbidity. As only samples that were absolutely clear were included in the study this precaution may also tend to lower the vitamin values reported in this paper.

Analytical technique

A blood sample of 50 ml was drawn in the morning from the fasting individual. Coagulation of the blood was prevented by potassium oxalate. After centrifugation 20 ml of plasma were measured into a 125 ml Erlenmeyer flask with a ground glass joint and 20 ml of 95% alcohol and 4 ml of 60% potassium hydroxide added. A water-cooled reflux condensor was inserted and the sample brought to boiling on an electric plate. After 15 minutes of boiling, the sample was cooled and transferred to a 125 ml separatory funnel containing 10 ml of water. The saponification flask was rinsed with 2 successive portions of 15 ml of water and 3 portions of 25 ml of ether, the washings being added to the sample in the separatory funnel. The funnel was agitated gently for 1 minute, after which the layers were allowed to separate. As a rule, less than 10 minutes were required for complete separation. After separation the lower, aqueous layer was drained off into another separatory funnel and re-extracted by shaking with 2 successive portions of 25 ml of ether, each ether sample after separation being added to the first ether extract. The combined ether extracts² were then washed in the separatory funnel with 2 successive portions of 15 ml of water. The separation of the ether layer from the water was usually accomplished in a few minutes; in those instances where the separation occurred more slowly the addition of a few milliliters of alcohol usually hastened the separation. After the last washing the ether was transferred to a 250 ml beaker

² It was found that treatment with 3 successive portions of ether sufficed for the complete extraction of the vitamin A from plasma samples to which enough vitamin A had been added to bring the vitamin concentration to above 300 μg %.

placed on an electric plate and the solvent evaporated by employment of gentle heating and constant application of a current of nitrogen from a cylinder. The residue was dissolved in petroleum ether and made up to 10 ml volume.

For determination of *total carotenes* the sample was compared in a Dubosque colorimeter with a standard carotene solution, containing 200 μg of β carotene in 100 ml of petroleum ether.

The $\alpha + \beta$ *carotenes* were determined by fluid chromatography and subsequent colorimetry as outlined above. For adsorption of the carotenes a column of aluminum oxide³ was prepared, approximately 50 mm long and 2.5 mm wide, through which 2.5 ml of the petroleum ether sample was passed under gentle suction. The elution of the $\alpha + \beta$ carotenes was performed by addition of 3–4 ml of a mixture of equal volumes of benzole and petroleum ether, which was passed through the column in 1 ml portions. The first of these portions usually contained all the eluted carotene, the subsequent samples being colorless. For estimation of the $\alpha + \beta$ carotene value the eluant was made up to a proper volume and compared in the Dubosque colorimeter with the above-mentioned carotene standard solution. The standard deviation of the $\alpha + \beta$ carotene measurements was found to be 10 to 15 μg %.

The determination of *vitamin A* was performed in duplicate on two 3.5 ml portions of the petroleum ether solution. The samples were pipetted into calibrated test tubes fitted for use in a photocolorimeter. The petroleum ether was evaporated in a water bath under application of a strong current of nitrogen. The residue was dissolved in 1.7 ml redistilled chloroform, after which 5 drops of acetic anhydride were added. After placing the tubes in the colorimeter 8.3 ml of a 25% solution of antimony trichloride in chloroform were rapidly added and the light transmission measured within a few seconds after the addition. The vitamin A value was

³ Alumina adsorption, Fisher Scientific Company.

obtained from a curve constructed by measurements on samples from a standard solution of vitamin A alcohol in chloroform.⁴ The vitamin A values obtained from the curve were corrected for the color yielded by carotene in the Carr-Price reaction, using the following formula (Hawk, Oser and Summerson, '47):

$A - \frac{C}{20} =$ micrograms of vitamin A per 100 ml of plasma, where A is the concentration of apparent vitamin A and C that of carotene, both expressed per 100 ml of plasma.

As it appears debatable whether the correction for total carotenes or $\alpha + \beta$ carotenes gives the truest vitamin A value both the corrected figures will be given in the report of the analyses.

The standard deviation of the vitamin A estimation was found to be 2.6 μg per 100 ml of plasma.

EXPERIMENTAL

In figure 1 the vitamin A values are plotted graphically in relation to the age of the individuals. Table 1 contains the summarized carotene and vitamin A values for the age groups 16-39, 40-59, 60-69, 70-79, and 80-89 years, and the values for the men and women separately.

It will be seen from the reported data that both the carotene and the vitamin A values show great variation in the different individuals. It will further be noted that the average concentrations of plasma total carotenes and $\alpha + \beta$ carotenes are higher (320 and 190 $\mu\text{g} \%$) in the younger individuals (16-39 years) than in the middle-aged and older groups. An analysis of the figures reveals, however, that the difference between the means is of doubtful significance, since it is smaller than the standard deviation of the difference.

⁴As solutions of the crystalline vitamin A alcohol in chloroform deteriorate quickly (in contrast to vitamin A in plasma extract preparations) the photoelectric determinations necessary for construction of the standard curve were performed immediately after the preparation of the solutions.

The average concentrations for vitamin A, total carotenes, and $\alpha + \beta$ carotenes in plasma were 17–23, 200–320, and 100–190 $\mu\text{g } \%$, respectively. No certain difference was found between the male and female subjects studied.

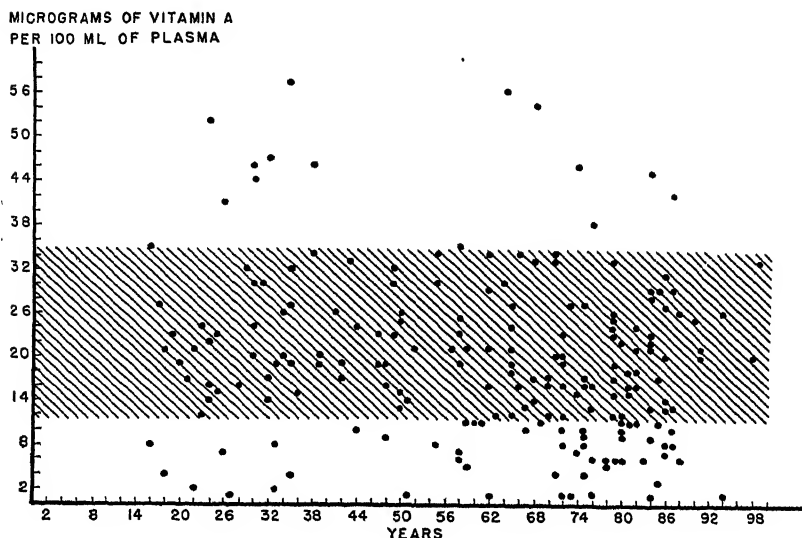


Fig. 1 The vitamin A values of plasma in 202 individuals. Correction for total carotenes was applied. The hatched area indicates values within one standard deviation from the mean observed in the younger age group.

SUMMARY

An investigation was made of the concentrations of plasma vitamin A, total carotene and $\alpha + \beta$ carotene in 155 middle-aged and old individuals offered a diet adequate in vitamin A, and in 47 younger subjects. The individual values observed varied greatly, but no statistically significant differences were found between the calculated means of the various age groups. The average concentrations of vitamin A, total carotenes, and $\alpha + \beta$ carotenes in plasma were 17–23, 200–320, and 100–190 $\mu\text{g } \%$, respectively. No certain difference was found between the male and female subjects studied.

TABLE 1

Total carotene, $\alpha + \beta$ carotene, and vitamin A values of plasma in 202 individuals

AGE GROUP	NUMBER OF INDIVIDUALS	TOTAL CAROTENES			$\alpha + \beta$ CAROTENES			VITAMIN A ²			VITAMIN A ³			
		Range of individual values	Mean value	S.D. ¹	Range of individual values	Mean value	S.D. ¹	Range of individual values	Mean value	S.D. ¹	Range of individual values	Mean value	S.D. ¹	
Micrograms per cent														
16-39	Total	47	120-540	320	91	70-380	190	77	1-57	23	13.4	3-64	30	14.7
	Male	22	120-540	320	113	70-380	190	94	1-57	23	13.0	3-64	30	14.2
	Female	25	170-460	320	70	70-300	180	61	2-47	22	13.6	7-58	30	15.0
40-59	Total	34	100-380	220	87	30-270	120	61	1-35	20	8.9	6-41	25	9.4
	Male	19	100-380	220	88	40-270	120	58	6-35	21	7.9	11-41	26	8.8
	Female	15	100-380	230	85	30-250	120	64	1-32	17	9.4	6-40	23	10.2
60-69	Total	25	70-320	200	67	20-210	110	53	1-56	22	12.8	3-61	27	13.6
	Male	15	70-310	190	74	40-210	120	56	1-56	22	15.7	3-61	27	16.3
	Female	10	110-320	200	61	20-160	90	41	12-33	21	6.8	14-41	28	8.6
70-79	Total	45	40-440	210	91	20-260	110	53	1-46	17	10.5	4-58	23	11.8
	Male	22	70-400	200	89	30-240	100	55	1-33	13	8.1	4-39	18	8.7
	Female	23	40-440	210	93	20-260	110	51	1-46	22	11.0	7-58	28	12.8
Above 80	Total	51	80-410	210	87	20-240	110	55	1-45	18	9.5	5-49	23	9.5
	Male	15	100-410	200	80	40-240	120	58	1-40	19	11.0	5-45	24	11.0
	Female	36	80-380	210	89	20-220	100	53	1-45	17	8.9	5-49	23	9.2

¹ Standard deviation.

² Correction applied for total carotenes.

³ Correction applied for $\alpha + \beta$ carotenes.

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STORAGE BY BOBWHITE QUAIL OF VITAMIN A FED IN VARIOUS FORMS

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Two forms of true vitamin A are found in animals, the alcohol that normally circulates in the blood stream, and the ester that comprises the bulk of vitamin A stores in the liver (Glover, Goodwin and Morton, '47). Other esters, including vitamin A acetate, do not occur naturally, but have been synthesized in the laboratory.

In the plant kingdom true vitamin A is non-existent, but instead there are certain carotenoid pigments that are converted to vitamin A by animals. Theoretically, 3 of these pigments, namely, alpha and gamma-carotene, and cryptoxanthin, are changed to vitamin A molecule for molecule; whereas a fourth, beta-carotene, will produce 2 molecules of vitamin A for each molecule of the pro-vitamin. Actually, such an efficient conversion of the carotenoids does not exist.

The biological value of carotene is much lower than that of vitamin A (Ewing, '41). Ahmad and Malik ('33) and Rosenberg ('45) pointed out that animals differ radically in their abilities to synthesize vitamin A from carotene. Kemmerer and Fraps ('38) maintained that not only does the utilization of carotene depend on the kind of animal involved, but also on the quantity of carotene fed and the nature of the carrier. Carotene in cottonseed oil, for example, was digested twice as efficiently by rats and chickens as carotene

in alfalfa leaf-meal. According to Russell ('39) greater assimilation of carotene takes place on a high-fat diet than on a low-fat diet.

Studies by Nestler ('46) have shown that the bobwhite quail should be included in the list of species utilizing vitamin A more efficiently than carotene. Quail maintained on an unsupplemented winter-holding diet had a survival rate equal to that of birds maintained on the same diet supplemented with 500 I.U. of carotene per pound ($\frac{1}{6}$ of the birds' requirements for optimum maintenance and subsequent reproduction), while the survival rate for birds on the basal diet supplemented with 500 I.U. of vitamin A was more than twice as great. The hatchability of eggs, and the survival of offspring were significantly higher for quail maintained on vitamin A-supplemented diets than for birds receiving an equivalent number of units of carotene. Also, the liver storage of vitamin A in both growing stock and birds being maintained through the winter on diets containing vitamin A and carotene in excess of body-requirements, was 3 to 15 times greater from the former than from the latter.

During the winter of 1945-46, the livers of 59 wild quail killed in 4 eastern states showed an average storage of vitamin A of over 300 I.U. per gram. Hence, several questions have arisen. Did this stored surplus come from carotene alone, or from a mixture of carotene and vitamin A? Would massive intakes of carotene promote storage of vitamin A in pen-reared quail to the same extent as that found in the wild birds? Would the precursor in a natural carrier like alfalfa leaves be assimilated by quail as efficiently as crystalline carotene in cottonseed oil? Can the 3 forms of true vitamin A, the alcohol, the natural ester, and the "synthetic" acetate, be utilized by quail from the diet to the same degree? During 1946-47, 3 experiments involving 236 quail chicks, were conducted at the Patuxent Research Refuge, Laurel, Maryland, to answer these questions.

PROCEDURE

The basal feed mixture used in these experiments had the following percentage composition: ground white corn 33.38, soybean oil meal 51.00, dried skim milk 10.00, butyl fermentation flavin concentrate 2.00, steamed bonemeal 1.86, ground limestone 0.56, salt mixture¹ 1.00 and vitamin D-activated animal sterol 0.20.

The experimental diets consisted of this basal mixture supplemented by the addition of vitamin A. Fresh batches of each experimental diet were prepared each week during the experimental period, and the vitamin A activity of each diet and of each supplement was determined spectrophotometrically at frequent intervals to insure that the levels of vitamin A intake remained constant. In experiment 1, each diet contained 3000 I.U. of vitamin (or pro-vitamin) A per pound of feed, the amount shown by Nestler ('46) as meeting the quail's requirements for maximum growth. In experiment 2, the diets contained 25,000 I.U. per pound, or more than 8 times the quantity required for maximum growth.

Five forms of the vitamin were compared. Diet 1 contained vitamin A ester from natural sources; diet 2, vitamin A acetate; diet 3, vitamin A alcohol; diet 4, crystalline carotene (90% beta-10% alpha); diet 5, a mixture of vitamin A ester and crystalline carotene, each of which provided half the units. Each form was dissolved in sufficient cottonseed oil to establish a potency of 2000 I.U. per gram of solution. These diets were fed ad libitum to pens of 18 or 19 quail chicks during the first 10 weeks of life.

Three diets were compared in experiment 3, each one being fed ad libitum to 12 birds for the first 10 weeks of life. Each mash contained 5000 I.U. of vitamin (or pro-vitamin) A per pound of feed: Diet 1, vitamin A acetate; diet 2, crystalline carotene in cottonseed oil; and diet 3, carotene of dehydrated alfalfa leaf meal.

¹ The salt mixture consisted of 100.0 parts of iodized sodium chloride plus 1.7 parts manganous sulfate.

At the close of each experiment birds were sacrificed, 6 from each diet of experiments 1 and 2, and 5 from each diet of experiment 3. The livers were assayed spectrophotometrically for vitamin A by use of a modification of the technique of Wilkie and DeWitt ('45), those of the first 2 studies as individual samples, but those of the last study as composite samples, one per diet. The data from experiments 1 and 2 were analyzed statistically by Fisher's ('32) "t" and "2" tests.

RESULTS

The results are summarized in table 1.

TABLE 1

*Storage of vitamin A in livers of growing quail at end of 10 weeks
(I.U. per gram of liver)*

EXP. NO.	LEVEL OF VIT. OR PROVIT. A (I.U./LB.)	AVE. AND RANGE OF STORAGE	FORM OF VITAMIN A					
			Vit. A ester	Vit. A acetate	Vit. A alcohol	Crystal- line carotene	$\frac{3}{2}$ Carotene Ester	Carotene of alfalfa leaf meal
1	3,000	Ave.	47	122	78	28	47	..
		Range	38-55	49-157	22-108	18-44	24-75	..
2	25,000	Ave.	2460	3966	1655	228	649	.
		Range	1084-	3038-	1471-	89-	323-	
			3536	5430	2096	532	926	
3	5,000	Ave.			244	.	24	52

Experiment 1

The differences between the storage from vitamin A acetate and that from any of the other supplements are highly significant. The difference between vitamin A alcohol and either the natural vitamin A ester or the combination of ester and carotene is according to odds of 19:1, whereas that between vitamin A alcohol and carotene is according to odds 99:1.

When the efficiency of utilization of crystalline carotene for storage by quail at the 3000 I.U. level in the diet is given the rating of 1, the other supplements receive the following

ratings: Combination of carotene and vitamin A ester, 1.7; vitamin A ester, 1.7; vitamin A alcohol, 2.8; and vitamin A acetate, 4.4.

Experiment 2

Again the difference between the storage from vitamin A acetate and that from any of the other supplements is highly significant. This time, however, the results on natural vitamin A ester are greater than those on vitamin A alcohol (odds of 19:1), or those from either carotene, or the combination of carotene and the ester (odds of 99:1). The difference between the storage from vitamin A alcohol and that from either carotene or the combination, is highly significant.

If, at the 25,000 I.U. level in the diet, carotene is rated as 1 for storage, then the other ratings would be as follows: Combination of carotene and vitamin A ester, 2.8; vitamin A alcohol, 7.3; vitamin A ester, 10.8; and vitamin A acetate, 17.4.

At the high level of vitamin A considered in this study, as much as 530 I.U. of the factor were stored per gram of liver from carotene by some quail during the first 10 weeks of life.

Experiment 3

The storage of vitamin A from the carotene of alfalfa leaf meal was nearly double that from the crystalline carotene in cottonseed oil. The storage from the vitamin A acetate, on the other hand, was over $4\frac{1}{2}$ times as great as that from the carotene of the alfalfa, and over 10 times as great as that from crystalline carotene.

DISCUSSION

Quail, after the first few weeks of life, are primarily granivorous and to some extent frugivorous. Handley's ('31) analysis of 1659 quail-crops disclosed the fact that only 14.5% of the food consisted of animal matter, mostly insects. According to Massey ('38) less than 5% of animal food is consumed by quail during winter months.

Analysis at Patuxent Research Refuge of 40 species of small animals that might be used as food by quail, revealed no evidence of vitamin A except in 4 cases, namely, earthworms, spiders, spittle bug larvae, and grain weevil larvae. However, in the light of additional tests, there is considerable doubt about even those 4 species possessing any true vitamin A. On the other hand, herbivorous small animals, such as grasshoppers, Colorado potato beetles, Mexican bean beetles, tent caterpillars, and the like, were found to have high concentrations of carotene, in some cases running as high as 230 I.U. per gram of entire carcass. Therefore, apparently the main, and possibly the sole, source of vitamin A for wild quail is carotene, obtained either directly from plants or indirectly from herbivorous insects and similar small animals.

SUMMARY

According to studies conducted with 236 bobwhite quail chicks at Patuxent Research Refuge, crystalline carotene in cottonseed oil fed at levels of 3000 I.U. (the requirement for optimum growth), 5000 I.U., and 25,000 I.U. per pound of feed, was utilized only $\frac{1}{3}$ to $\frac{1}{7}$ as efficiently as vitamin A alcohol; $\frac{1}{2}$ to $\frac{1}{10}$ as natural vitamin A ester; and $\frac{1}{4}$ to $\frac{1}{17}$ as vitamin A acetate, based on the storage of vitamin A in the liver.

The carotene in the natural carrier, alfalfa leaf meal, was assimilated as effectively as was crystalline carotene in cottonseed oil, when both were fed at 5000 I.U. per pound of feed.

Crystalline carotene when fed at a level over 8 times the requirement of the quail for maximum growth, or 25,000 I.U. per pound of feed, was stored as vitamin A in the livers of pen-reared quail to an extent comparable to the levels found in certain young wild quail.

Vitamin A acetate was utilized more efficiently by quail than either vitamin A alcohol or the natural vitamin A ester.

There was considerable individual variation in storage of vitamin A by quail on the same diet, and with the same or similar parental background.

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IMPLANTATION IN NORMAL AND VITAMIN E DEFICIENT RATS ¹

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TWO FIGURES

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Whether vitamin E is essential for the capacity of the rat to become pregnant is a controversial question. In previous work, the influence of alpha-tocopherol acetate on the implantation rate of old rats had been studied (Kaunitz and Slanetz, '47). Experiments concerned with the influence of vitamin E deficiency upon implantation during the entire life of the rat are reported below.

METHODS

A highly inbred colony of albino rats was maintained on the vitamin E deficient diet given in table 1. The tocopherol content of this diet, estimated by an internal standard method (Kaunitz and Beaver, '44), was from 0.2 to 0.4 mg per 100 gm of diet, affording a daily intake of about 20–40 μ g to an adult rat.² The controls received the same diet supplemented by 3 mg synthetic dl-alpha-tocopherol acetate per 100 gm diet, allowing a daily intake of approximately 300 μ g.³ Rats of the

¹ Aided by a grant from the John and Mary R. Markle Foundation.

² We are indebted to Drs. Philip L. Harris and Mary L. Quaife of the Distillation Products, Inc. for their help in the examination of lard samples.

³ Dr. Leo Pirk of Homann-LaRoche, Inc., kindly supplied us with the synthetic dl-alpha-tocopherol acetate and the other vitamins used.

1st to 8th generations, inclusive, raised on this diet were used. Some of the rats had received 1 or 2 doses of 1-3 mg of alpha-tocopherol acetate several months before they were used in the mating experiments recorded in figure 2.

Mating was carried out by leaving the female with the selected male for 5 days. On and subsequent to the 15th day after mating had begun, the animal was repeatedly inspected grossly for the placental sign,⁴ and its weight was taken

TABLE 1
Composition of vitamin E deficient diet

BASAL MIXTURE		SUPPLEMENTS OF BASAL MIXTURE	
	%		mg/kilo
Casein, crude	30	Thiamine chloride	2
Cerelose	54	Riboflavin	4
Lard, commercial	10	Pyridoxine	4
Salt mixture (Hawk-Oser) ¹	4	Calcium pantothenate	10
Celluration	2	p-Amino benzoic acid	300
		Choline	1000
		Inositol	1000
		Vitamin K	4
		Oleum percomorphum	200

¹ Hawk, P. B., B. Oser and W. H. Summerson 1947 Practical Physiological Chemistry, 12th Edition. See page 1273. The Blakiston Co., Philadelphia.

daily. A positive placental sign accompanied by a gradual weight gain followed by weight loss was taken as proof of a resorption gestation. If the placental sign was persistently absent, and if the weight was constant, the absence of pregnancy was recorded.⁵ The results obtained in this manner

⁴ The "placental sign" refers to the appearance of blood in the rat's vagina 13-14 days after a mating. It is caused by the development of placental tissue.

⁵ Some of the rats were used for matings after a previous resorption gestation or normal one had occurred, but at least 4 weeks were permitted to elapse after the resorption gestation or the weaning of the young. Contrary to the experiences of A. L. Bacharach and E. Allchorne (Biochemical Journal, 1938, 32: 1298), we did not observe a drop in the implantation rate of animals that had previously had either a resorption or normal gestation, if animals of the same age were compared with virginal rats. This difference may be due to the fact that in Bacharach and Allchorne's experiments, the second mating was done 5-8 days after the termination of the first gestation.

were not always beyond question, particularly in rats below 2 and above 7 months of age. Therefore, laparatomies were carried out in more than 100 cases, and the uteri grossly examined for implantations. The answer disclosed by the laparotomy coincided in more than 90% of the cases with the previous "clinical diagnosis." We are therefore confident that the number of errors which may have occurred in the tests not checked by laparotomy was insignificant. Some

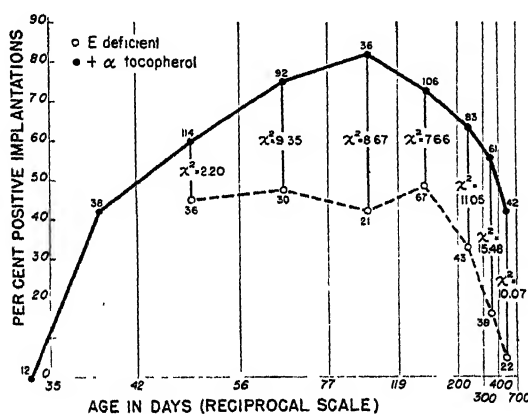


Fig. 1 Implantation rate in rats on a purified vitamin E deficient diet and on the same ration supplemented by 3 mg dl-alpha-tocopherol acetate per 100 gm diet. The numbers close to the circles indicate the number of experiments. Chi square denotes the probability factor.

pseudopregnancies may have been registered as resorption gestations, but the avoidance of this error would only have enhanced the significance of the differences.

The males used for the matings were derived from the same colony. They were raised on the deficient diet supplemented by 3 mg alpha-tocopherol acetate per 100 gm of diet. A negative mating test was accepted for the charts only if the male had proved to be fertile before and after the negative test; the weights were taken at intervals of 1 to 2 weeks and they were used only if no weight losses had occurred.

EXPERIMENTAL

In figure 1 are recorded the results of the mating experiments with rats on the vitamin E deficient diet and the same diet supplemented by alpha-tocopherol acetate. No implantations were observed among the animals on the diet supplemented by alpha-tocopherol acetate before the 35th day; thereafter, the rate of implantation went up steeply. This agrees with observations on the occurrence of the first estrus in rats (Engle et al., '37), and their attainment of sexual maturity (Blandau, '43). At the age of approximately 3 months, the implantation rate reached about 85%, which agrees with the results of Evans and Burr's ('27) studies on the implantation rate in a normal rat colony. Thereafter, the rate declined; but, even at the age of 18 months, nearly half of the rats on the tocopherol supplemented diet became pregnant.

In the deficient group, the implantation rate ran consistently lower than that of the controls. After the 9th week, the differences were statistically significant.⁶

In figure 2 are given the individual results of the mating tests and the body weights of the vitamin E deficient rats above the age of 150 days. One group had received 1 or 2 oral administrations of 1-3 mg alpha-tocopherol several months before the matings were recorded; the second group had been given no supplements. The administration of the tocopherol supplements several months before these experiments increased the body weights and the implantation rate. Implantations in the group without supplement were very rare after the 7th month, which is in good agreement with results of Emerson and Evans ('39).

⁶ The chi square was calculated according to George W. Snedecor (Ames, Iowa, 1940). If chi square is greater than 3.8 the probability of an error is below 5%. We are indebted to Drs. Theodore F. Zucker and Lois Zucker for their help in the statistical analysis of the results.

DISCUSSION

Neither dietary deficiencies other than that of vitamin E nor infections can explain the low implantation rate in the vitamin E deficient rats. Dietary deficiencies other than of vitamin E can be ruled out because addition of alpha-tocopherol to the basic diet increased the implantation rate to normal. Infections of the genital organs may have occasionally

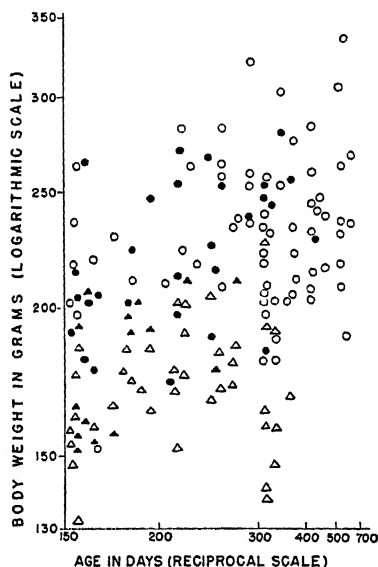


Fig. 2 Implantation rate in vitamin E deficient rats. The circles indicate rats which had received 1-3 oral supplements of 1-3 mg alpha-tocopherol acetate several months before the experiment was recorded. The triangles refer to rats without tocopherol supplements. Full circles or triangles denote pregnancy; hollow circles or triangles signify absence of implantation.

interfered with implantation in older animals; but they were not responsible for implantation failure in most cases because single tocopherol doses administered after mating increased the implantation rate significantly (Kaunitz and Slanetz, '47). Moreover, infections of the genital organs in deficient rats under 5 months of age are extremely rare. It seems, therefore, very probable that the effect of tocopherol

on the implantation rate is specific and not the result of secondary factors.⁷

Emerson and Evans ('39) as well as Goettsch and Pappenheimer ('41), found normal implantation rates in young rats on a vitamin E deficient diet. In the experiments of these authors, a diet was used which contained at least 20% lard and 8-12% dried yeast. These diets very probably permitted a daily intake of 40-80 μ g of tocopherol for an adult rat in contrast to the 20-40 μ g per day afforded by the diet used in our experiments. In view of the fact that a daily intake of 55 μ g is, under certain conditions, on the border line of the requirements for normal gestations (Mason and Filer, '47), the tocopherol contents of some experimental diets may well have obscured the effect of tocopherol on implantation. Even with 20-40 μ g, about 50% of the younger rats became pregnant. It is, however, extremely dangerous to compare tocopherol requirements in experiments carried on with widely differing diets because the vitamin E needs are greatly influenced by the quantity and the quality of many dietary constituents, especially the fats. This has recently been stressed by Mason and Harris ('47).

The beneficial effect on subsequent growth and implantation in female rats of single doses of 1-3 mg of tocopherol administered early in life resembles the influence of single tocopherol doses on growth and testicular development in males (Kaunitz, '46; Kaunitz et al., '44).

⁷ We are indebted to Dr. Charles E. Tobin of the Department of Anatomy, University of Rochester, for this communication:

"An inbred strain of black mice obtained from Dr. Strong at Yale and known as the C-57 strain were started on a vitamin E-deficient diet (diet 69) on the day of delivery. If the females from these litters are mated with normal males between 75 and 100 days of age they will become pregnant and resorb their young. This fetal resorption can be prevented by oral administration of tocopherols or implantation of various tocopherol esters in the form of pellets subcutaneously. However, if such E-deficient females are kept on the E-deficient diet for 100 to 150 days or more before mating with normal males, pregnancy will not ensue although copulation plugs or sperm will be found in the vaginae of these animals for several successive estrous cycles. I have observed this phenomenon in six mice which were studied especially for this purpose although it has occurred in other animals previously, and is being found in more animals now under observation."

Storage and gradual utilization of the single dose of 1-3 mg of tocopherol can hardly explain the effects lasting for at least 1 year because this would have provided only 3-10 μ g per day, which amount is within the limits of error of dietary intake. A valid explanation for this effect has not as yet been given. The protracted effect of single doses of tocopherol administered early in life makes more difficult the calculation of "daily" requirements.

In figure 2, it can be seen that no mating experiments were carried out on rats older than 1 year in the group without tocopherol supplements. Ten experiments on "single dose" animals over 500 days old were recorded. This was possible because the life span of a rat given a single supplement before the 5th month is considerably longer than that of an animal without such a supplement. Whether or not this is a specific tocopherol effect is not clear; it may be indicative of a higher resistance to respiratory and genital infections in the protected group.

The mechanism of the implantation failure in vitamin E deficiency is not known. Experiments dealing with this question are being carried out in cooperation with Dr. Richard J. Blandau of the Department of Anatomy, The University of Rochester School of Medicine and Dentistry.

SUMMARY

1. The implantation rate in a highly inbred rat colony maintained on a complete, purified diet was determined until the rats were 2½ years old.

2. The implantation rate of rats maintained on the same diet without the tocopherol supplement was found to be significantly lower than that of the control group after the rats were 9 weeks old. Tocopherol is essential for the ability of the rat to become pregnant.

3. Single doses of 1-3 mg of alpha-tocopherol administered early in life have a beneficial effect on subsequent growth, implantation rate, and life span of the female rat.

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CHOLINE DEFICIENCY IN THE BABY PIG

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TEN FIGURES

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In previous papers it has been shown that day-old pigs can be raised to weaning age on a completely "synthetic" diet made up to simulate cow's milk (Johnson, James and Krider, '47, '48). With such a synthetic diet, it is possible to omit or alter 1 or more constituents at a time, thus making possible investigation of the nutritional requirements of the baby pig. Such information should be useful in studies on human infant nutrition.

In this paper we wish to report the production of a choline deficiency in baby pigs by omitting choline from the synthetic milk ration.

Wintrobe, Miller, Follis, Stein, Mushatt and Humphreys ('42) fed 3- to 4-month-old pigs a choline-deficient diet and observed an increased amount of fat in the livers and some abnormality of gait, but found no effect on growth. Ellis, Madsen, and Miller ('43) reported that choline is necessary in addition to pantothenic acid and pyridoxine for protection from locomotor incoordination resulting from nerve degeneration, but that growth is not affected. Ensminger, Bowland, and Umha ('47) fed sows a choline-low ration and found that most of the baby pigs from these sows failed to survive.

EXPERIMENTAL

Nine pigs, 6 1-day-old Chester White pigs from 1 litter and 3 4-day-old Duroc-Jersey pigs from another litter, were

divided into 3 groups consisting of 1 Duroc-Jersey and 2 Chester White pigs in each group. Group 1 was fed the "complete synthetic milk" ration given in table 1. Group 2 was fed the same ration with the choline, inositol and *p*-aminobenzoic acid omitted; and group 3, the complete ration with

TABLE 1
Composition of "synthetic milk" ration

	%
Casein (Labco, vitamin-free)	30
Glucose (cerelose)	37.4
Mineral salts ¹	6
Lard	26.6

Made up and homogenized into a milk containing 13% solids including 4% lard (liquid basis).

The following vitamins ² were added to the complete diet per 1000 gm of milk:

Thiamine	0.65 mg	<i>p</i> -Aminobenzoic acid	2.6 mg
Riboflavin	1.30 mg	Pteroylglutamic acid	0.052 mg
Pyridoxine	1.30 mg	Biotin	0.01 mg
Calcium pantothenate	7.8 mg	α -Tocopherol acetate	1.0 mg
Inositol	26.0 mg	2 methyl-1,4-naphthoquinone	0.26 mg
Choline	260.0 mg	Vitamin A	1000 I.U.
Nicotinic acid	2.6 mg	Vitamin D ₂	100 I.U.

¹ See Johnson et al. ('48).

² The thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, biotin and α -tocopherol acetate used in this experiment were very generously supplied by Hoffmann-La Roche, Inc., Nutley, New Jersey, through the courtesy of Dr. J. C. Bauernfeind.

Pteroylglutamic acid was generously supplied by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York.

Inositol was generously supplied by the A. E. Staley Manufacturing Co., Decatur, Illinois.

choline omitted. All pigs were fed sulfathalidine ¹ at 2% on the dry basis to inhibit intestinal synthesis. The pigs were housed and fed ad libitum as previously reported (Johnson et al., '48).

The lard used in this ration was analyzed for choline by the Neurospora procedure of Horowitz and Beadle ('43) and

¹ Sulfathalidine (phthalylsulfathiazole) was generously supplied by Sharpe and Dohme, Philadelphia, Pennsylvania, through the courtesy of Dr. S. F. Scheidv.

was found to contain 10.6 γ of choline per gm, which equals 2.4 γ /ml for the "synthetic milk" ration. One sample of sow's milk was found to contain 273 γ /ml. Engel ('43) gives 147 γ /ml for the choline content of cows' milk.

The smallest of the Chester-White pigs weighed only 900 gm when started on experiment and died on the 2nd day, leaving only 2 pigs (1 Chester-White and 1 Duroc-Jersey) in the positive control group. The rest of the pigs were kept on the experiment for 8 weeks without further loss. During this experimental period blood samples were taken 4 times (at 2-week intervals), and hemoglobin concentration and red blood cell and white cell counts were determined.

At the end of the 8-week experimental period liver biopsies were taken of all animals.² One small piece from each liver was frozen immediately, sectioned with the freezing microtome, and stained with Sudan IV and light green.³ Another small piece was fixed, sectioned by the paraffin method, and stained with Harris's hematoxylin and Orange G.

RESULTS AND DISCUSSION

The growth curves of the experimental pigs are plotted in chart 1. Due to the marked difference in weights of the Duroc-Jersey as compared to the Chester-White pigs, the growth data are plotted as per cent of initial weight. Since there was no difference ($P=0.79$) in percentage gains of the pigs in group 2 and group 3 (choline-, *p*-aminobenzoic acid-, inositol-low vs. choline-low), they are treated as 1 choline-deficient group, and the 2 curves given are the averages of the percentage gains of the control and of the choline-deficient groups. The points plotted, however, are the individual data for each animal. In terms of actual weights, the Duroc-Jersey pig in the positive control group reached 24.55 kg (54 lbs.) at 8 weeks (i.e., good growth).

² We wish to express our appreciation to Dr. L. E. Boley and Dr. H. Hardenbrook for performing the operations.

³ We wish to express our appreciation to Prof. F. B. Adamstone of the Department of Zoology for sectioning and preparation of the slides from the frozen material.

Statistical treatment of the growth data gives the following probabilities: control group vs. group 2 (minus choline, inositol, *p*-aminobenzoic acid) $P=0.03$ (Fisher, '44); control group vs. group 3 (minus choline) $P=0.05$; control group vs. groups 2 and 3 (both groups minus choline) $P=<0.01$; while for group 2 vs. group 3, $P=0.79$. That is, the omission of choline from the "synthetic milk" ration of these baby

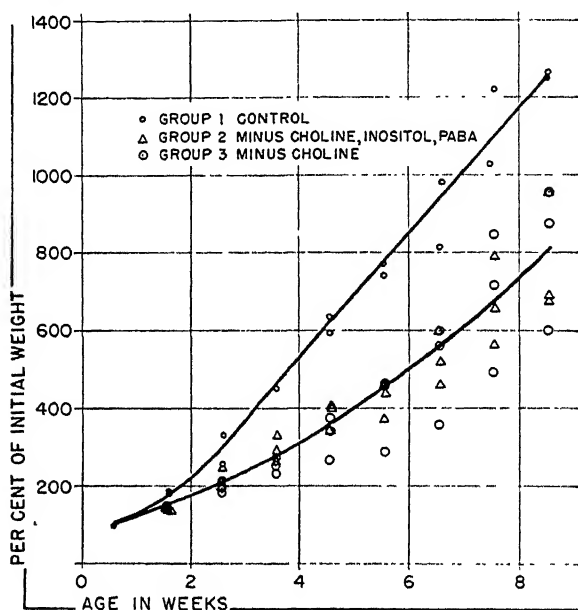


Chart 1 Growth curves of baby pigs on "complete" vs. choline-low "synthetic milk" diet.

pigs markedly retarded their growth. Plate 1 shows the pictures of pigs 2 and 3 (positive controls, group 1) and 7, 8, and 9 (minus choline, group 3) at 5 weeks. The differences in size of these pairs, which initially weighed approximately the same, are quite evident. As well as difference in size, the choline-deficient pigs presented in some cases a generally unthrifty appearance and rough haircoat.

The hemoglobin values and red blood cell counts are given in table 2. No significant differences between groups with

respect to hemoglobin concentration or white cell counts were found. However, the increases in red blood cell count during the 8-week experimental period did show significant differences. The control group showed an average increase of 2.37 million cells per mm^3 , while the choline low groups showed an average increase of only 0.86 million cells per mm^3 ($P = 0.044$).

TABLE 2

Red blood cell counts and hemoglobin concentrations for baby pigs fed the "synthetic milk" rations

GROUP	WEEKS ON EXPERIMENT			
	2	4	6	8
<i>Average red blood cell counts in millions/mm³</i>				
1 (control)	6.64	7.78	8.22	9.02
2 (minus choline, inositol and <i>p</i> -aminobenzoic acid)	6.72	6.39	7.63	7.61
3 (minus choline)	6.54	6.90	7.45	7.37
<i>Average hemoglobin concentration in gm/100 ml</i>				
1	13.3	13.8	11.8	13.2
2	11.5	10.5	12.3	12.4
3	10.9	10.2	11.4	11.8

The liver sections taken by biopsy were examined histologically, and plate 2 illustrates the results found in each group of baby pigs. As can be seen, there was severe fatty infiltration in the livers of the pigs from both groups 2 and 3, while the livers from the control group were normal in appearance. There appears to be somewhat more fat deposition in the livers of the pigs in group 2 (minus choline, inositol and *p*-aminobenzoic acid, plate 2, figs. 6 and 9) than in the livers from group 3 (minus choline, figs. 5 and 8), which may indicate some role of inositol in fatty liver formation in the baby pig. However, this must be further investigated on a greater number of pigs before a definite conclusion can be reached.

SUMMARY

One and 4-day-old pigs have been raised on a "synthetic" diet made up to simulate milk.

On this diet, which contains 30% protein as casein (dry basis), the baby pig has been found to require choline. When choline was omitted from the diet, the pigs gained weight at a slower rate and developed fatty infiltration of the liver. The pigs were fed ad libitum. The choline-deficient pigs did not show as good erythrocyte formation, as determined by red blood cell count increases during the 8 weeks to weaning, as did baby pigs receiving the "complete synthetic milk" diet. Definite evidence was not obtained for a requirement of inositol or *p*-aminobenzoic acid when their combined deficiencies were superimposed on a choline deficiency, although their omission from the diet appeared to accentuate the degree of fatty infiltration of the livers.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

Photographs of pigs after 5 weeks on experiment

- 1 Shows pig 2 on the "complete" diet and pig 7 on the choline-low diet.
- 2 Shows pig 2 on the "complete" diet and pig 8 on the choline-low diet.
- 3 Shows pig 3 on the "complete" diet and pig 9 on the choline-low diet.



PLATE 2

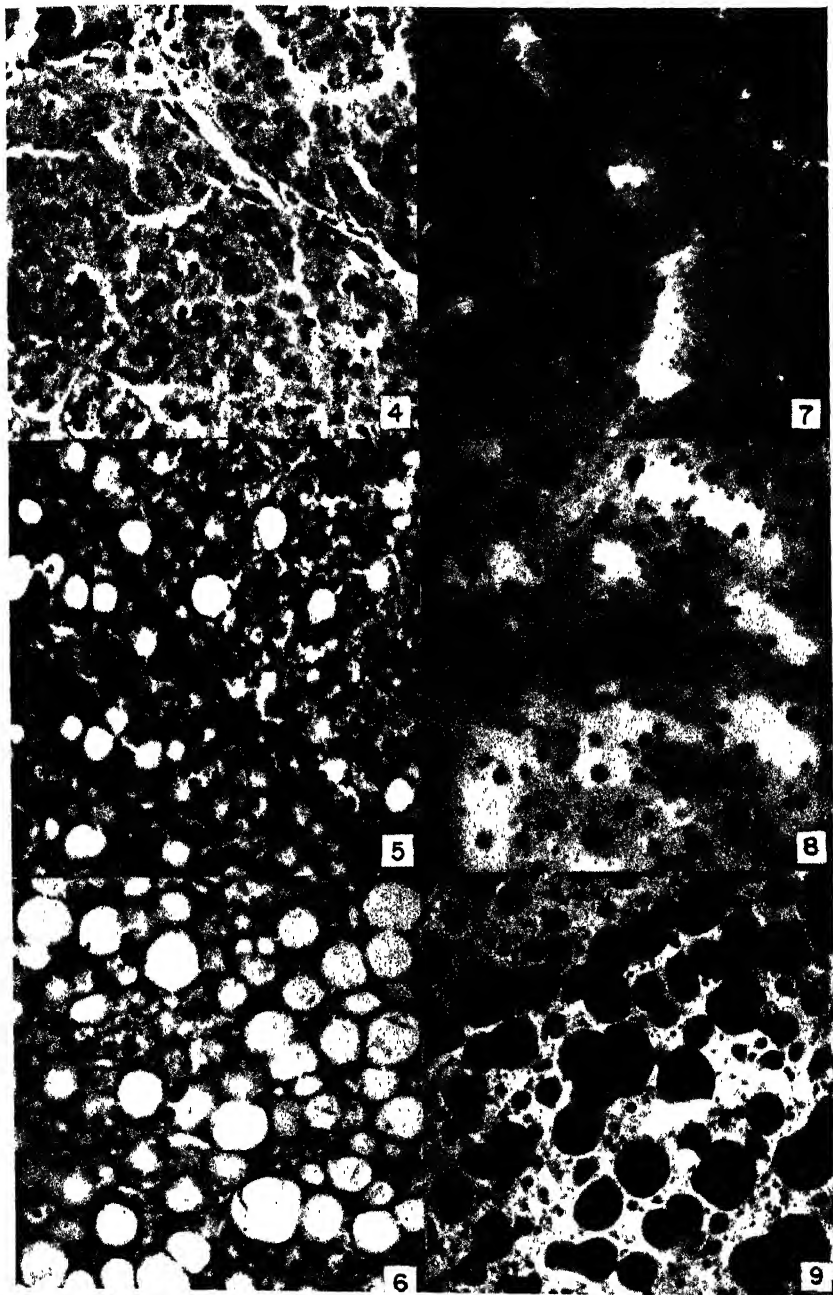
EXPLANATION OF FIGURES

Photomicrographs of liver sections of pigs at termination of experiment (8 weeks). Magnification $\times 225$ (original $\times 300$ reduced $\frac{1}{4}$). 4, 5, and 6 are paraffin sections stained with Harris's hematoxylin and Orange G, 7, 8, and 9 are frozen sections stained with Sudan IV and light green. Tissues in figures 7 and 8 were left frozen for some time before sectioning and hence some deterioration of tissue is shown by light spaces.

4 and 7 Liver from group 1 control (pig 2). Shows normal appearance and normal distribution of fat.

5 and 8 Liver from group 3, choline omitted (pig 8). Some fat infiltrations. Figure 5 shows fat removed in process of making paraffin section. Fat droplets are stained with Sudan IV in figure 8.

6 and 9 Liver from group 2, choline, inositol, and *p*-aminobenzoic acid omitted (pig 4). Note the large amount of fat infiltration.



POTASSIUM REQUIREMENT OF THE CHICK¹

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ONE FIGURE

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It has long been known that potassium plays a vital role in animal physiology. In spite of this, knowledge concerning the quantitative requirements of animals for this element is still in an unsatisfactory state. To a lesser extent this is also true of the deficiency symptoms which appear when suboptimum amounts of potassium are fed. Most of the work in this connection has been done with the rat and considerable variability in results has been reported. The earlier work with this species has been clarified to some extent by the recent report of Kornberg and Endicott ('46) who described the following symptoms in rats fed diets very low in potassium: prompt failure of growth, edema, multiple lesions in many tissues, and death. A dietary potassium level of 0.17% was found to be adequate for growth and the prevention of lesions.

While studying potassium deficiency in dogs, Ruegamer, Elvehjem and Hart ('46) observed symptoms which included cessation of growth, "stiff neck" and paralysis of the limbs, discolored teeth and hemoconcentration. Hughes and Ittner ('42) have reported that young pigs require approximately 0.15% of potassium in the diet for optimum growth. Apparently the only report of potassium deficiency in the chick is that of Ben Dor ('41). He observed retarded growth and

¹ This work was supported in part by a grant from the International Minerals and Chemical Corporation, Chicago, Illinois.

high mortality in chicks fed a low-potassium diet and concluded that at least 0.17% of potassium in the diet was necessary for maximum growth.

The potassium requirement of chicks has been reinvestigated in this laboratory in connection with a general study of factors affecting mineral metabolism in this species. Such a study was prompted by the limited information available and the need for a better characterization of the symptoms of potassium deficiency. Furthermore, unpublished results had been obtained which suggested a relationship between the potassium and phosphorus requirements of chicks. This possibility was also given further attention in the experiment reported here.

EXPERIMENTAL PROCEDURE

In this experiment it was desired to study both the potassium requirement and the possible relationship between this requirement and the amount of phosphorus present in the diet. Accordingly, the 7 different levels of potassium shown in table 1 were each fed to lots of chicks receiving 0.4% and 0.6% phosphorus, respectively. This smaller amount, 0.4%, is near the generally accepted minimum requirement for chicks while 0.6% is more nearly representative of an optimum allowance of this element. The 2 groups of chicks served not only to test the relationship between phosphorus and potassium requirements, but also as a double check on the quantitative requirement for potassium.

Except with respect to potassium, the basal diet used in this experiment was adequate in all nutrients known to be required by the chick. The diet of the lots receiving 0.4% phosphorus had the following percentage composition: corn-starch 61.55, blood fibrin 25, gelatin 5, cellophane 2, liver "L" 1, soybean oil 2, dicalcium phosphate 1.5, salts 1.45² and

²Salts (per cent of diet): CaCO_3 , 0.75; NaCl , 0.60; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.06; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.03; ZnCl_2 , 0.001; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.0003; NaI , 0.003; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.0017.

vitamins 0.5.³ The diet of the lots receiving 0.6% phosphorus was identical except that the dicalcium phosphate was increased sufficiently to raise the phosphorus content to 0.6% and the cornstarch was decreased accordingly. A calcium to phosphorus ratio of 1.9:1 was maintained in both instances by the addition of calcium carbonate. The basal diet contained 0.04% potassium as determined by analysis with the flame photometer.⁴ The desired potassium levels were obtained by adding a solution of analytical grade KCl to the basal mixtures.

White Leghorn male chicks were used as experimental animals. Lots of 15 1-day old chicks were placed in electrically heated battery brooders and supplied the experimental diets *ad libitum*. During the 4 weeks' experimental period the chicks were observed for symptoms of potassium deficiency and post mortem examinations were made of the chicks which died. Individual weights were taken at weekly intervals. At the end of 4 weeks all surviving chicks were sacrificed, further observations were made on the deficient lots, and standard bone ash determinations were carried out on representative chicks from each lot.

RESULTS AND DISCUSSION

The results obtained with respect to growth, bone ash, and mortality are presented in table 1. The striking effect of potassium on growth, calcification and viability of chicks is evident from these data. In the case of the chicks receiving the marginal level of phosphorus, growth was increased progressively by additional potassium up to a level of 0.24% of the diet. For the chicks receiving the higher level of phosphorus growth was similarly increased by additions of potassium up to a level of 0.20%.

³ Vitamins (mg per 100 gm): thiamine 0.3; riboflavin 0.5; calcium pantothenate 1.1; pyridoxine 0.5; niacin 1.75; vitamin K 0.22; folic acid 0.33; biotin 0.026; vitamin A alcohol 0.11; PABA 11.0; inositol 111; choline 222; tocopherols in oil 15; vitamin D₃ 50 AOAC chick units.

⁴ Dr. C. J. Barton made the analyses.

Calcification, as measured by the percentage of ash in fat-free, dry bone, was likewise benefited by supplementary potassium in the diet. In the case of the low phosphorus group, 0.20% potassium gave optimum bone ash values, and for the group receiving the higher phosphorus diet 0.16% potassium was adequate for optimum calcification. According to Cook and Robertson ('40), tibiae from day-old chicks contain approximately 36% ash. The percentage of bone ash found in chicks which received 0.08% potassium and 0.4% phosphorus represents a considerably lowered ratio of mineral matter to soft tissue deposited during growth. The poor

TABLE 1

The effect of different levels of potassium on growth, calcification, and mortality in chicks

LEVEL OF POTASSIUM	GROUP 1 (0.4% Phosphorus)			GROUP 2 (0.6% Phosphorus)		
	Wt. at 4 wks.	Bone ash	Mortality	Wt. at 4 wks.	Bone ash	Mortality
%	gm	%	%	gm	%	%
0.04	100	.	.	100
0.08	85	23.6	87	147	41.3	87
0.12	185	38.3	33	221	43.0	13
0.16	298	41.6	0	322	47.8	7
0.20	311	43.4	0	368	46.7	0
0.24	332	42.8	0	344	46.7	7
0.50	334	43.9	0	357	47.6	0

calcification found in the low potassium lots, therefore, cannot be accounted for on the basis of retardation of growth alone.

The sparing action of phosphorus on the potassium requirement as measured by both growth and calcification points to an interrelationship in the metabolism of these 2 elements. In view of the fact that growth and calcification were better in the lots receiving 0.6% phosphorus than in those receiving 0.4%, the latter level of dietary phosphorus is probably too low for the rapidly growing chick.

These results indicate that the potassium requirement for maximum growth of the chick is 0.20-0.24% which is

somewhat higher than the 0.17% previously reported by Ben Dor ('41). This difference is probably due to different experimental conditions. The conclusions drawn from the earlier work were based on growth response to a diet which permitted only relatively slow growth even when supplemented with potassium. It is probable that the much faster rate of growth obtained in the experiment reported in this paper increased the potassium requirement. The fact that some chicks used by Ben Dor ('41) were maintained on an adequate commercial diet for a period before receiving experimental treatment may also have contributed to the difference in results obtained.

Continuous observations were made of the behavior and deficiency symptoms of the chicks in this experiment. Retarded growth and development were the most immediate symptoms of an inadequate potassium intake. The growth of all chicks receiving suboptimum amounts of potassium was retarded throughout the experiment. Chicks receiving the basal diets made virtually no gains in weight even though some of them survived for as long as 24 days. Mortality began by the 5th day in lots receiving the lower levels of potassium. The general inferiority of these lots was clearly evident by the end of the 1st week, and became progressively more marked (fig. 1). In potassium deficient lots many chicks gradually lost the use of their legs and were unable to stand or walk. Pasting around the vent and the excretion of excessive amounts of urates were common in deficient lots. Feed consumption by deficient chicks was reduced, but even those in the basal lots maintained an active interest in food.

Death in deficient chicks was generally preceded by difficulty in breathing, sometimes for several hours, and the heart rate, normally very high in the chick, was markedly slower. Tonic spasms preceding death were also common. During these spasms the legs were rigidly extended and frequently the head was retracted. The leg and other body muscles were sometimes seized with tremors. Rigidly extended legs at time of death were very characteristic. This inability of the

muscles to relax is not surprising in view of the known function of K^+ ions in reducing muscular contractility and favoring relaxation in contrast to Ca^{++} ions which favor contraction.

Characteristic post mortem findings included emaciation, enlarged gall bladder, and swollen kidneys with deposits of urates in the ureters and sometimes throughout the kidneys. In some cases the kidneys were twice the normal size. The intestines and ceca were frequently distended by material collected in them. Less common findings included hydropericardium, discoloring lesions on the surface of the liver, and thin, eroded areas in the intestinal walls.



Fig. 1 Comparison of potassium deficient and normal chicks at 2 weeks of age. The chick on the left received 0.04% potassium while the one on the right received 0.20% potassium.

SUMMARY

1. Rapidly growing, White Leghorn male chicks were found to require from 0.20–0.24% dietary potassium for maximum growth. The requirement for potassium was slightly higher when the phosphorus in the diet was at a marginal level than when an optimum amount of phosphorus was present; 0.16% potassium was adequate for the prevention of mortality.

2. Chicks receiving a marginal level of phosphorus in the diet required approximately 0.20% potassium for optimum calcification of the bones, while the corresponding require-

ment in the case of chicks receiving an optimum allowance of phosphorus was 0.16% potassium.

3. Symptoms of potassium deficiency in the chick included retarded growth, weakness, loss of use of legs and the excretion of large amounts of urates. Death was preceded or accompanied by tetanic seizures in which the muscles were unable to relax. Mortality commenced as early as the 5th day in deficient chicks, and no chicks receiving the basal diet, containing 0.04% potassium, survived the 4 weeks' experimental period. Post mortem examination revealed lesions in several organs, particularly the kidneys and ureters which were enlarged and usually congested with urates.

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AVAILABILITY OF AMINO ACIDS IN SOME FOODS¹

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WITH THE TECHNICAL ASSISTANCE OF SHIRLEY DIETERICH,

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The nutritive value of a protein is dependent not only upon its content of essential amino acids (Rose, '38) but the biological availability of these amino acids as well. Incomplete digestion and absorption of an essential amino acid has the result of altering the effective composition of a food protein. The ultimate synthesis of body protein from the amino acids made available through digestion and absorption is dependent on a third factor, namely, the simultaneous appearance in the blood stream of each of the essential amino acids in suitable proportions. The reports of Elman ('39), Melnick, Oser and Weiss ('46), and Geiger ('47) emphasize that protein synthesis does not occur unless a complete mixture of the essential amino acids is present at one time.

Only limited data are to be found in the literature concerning the availability of amino acids in foodstuffs. That the availability of the individual amino acids might vary would be inferred from *in vitro* digestion studies (Mitchell and Hamilton, '29; Jones and Gersdorff, '33; Melnick, Oser and Weiss, '46) which indicate that amino acids are liberated from proteins at different rates characteristic of the amino acid or its

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linkage in the protein. In addition, it has been demonstrated (Chase and Lewis, '34) that specific amino acids are absorbed into the blood at different rates.

On the basis of sulfur balance studies with chicks, Evans and McGinnis ('47) concluded that the availability of cystine and methionine in soybean oil meal can be modified by the method of processing the meal. Methionine balance studies reported by Melnick et al. ('46) showed that 49% of the methionine in a sample of soybean oil meal appeared in the feces and hence was not available to the rat.

In the present investigation, the availability of all 10 of the essential amino acids in several foodstuffs was determined. Calculations were based on determination of the amino acids in the food and in the feces. Corrections for the metabolic amino acids in the feces were calculated from control periods in which a low protein egg ration was fed.

EXPERIMENTAL

The experimental techniques used in conducting the feeding tests were largely adaptations of the methods used by Mitchell and coworkers ('24, '26) in their investigations on the digestion and metabolism of proteins in foodstuffs. Groups of 5 rats each, weighing initially from 55 to 60 gm, were fed for a 24-hour period a stock ration containing 2% Fe_2O_3 as a feces marker. They were then fed ad libitum for 7 days an experimental ration which contained about 10% protein from a single source. Following this feeding they were again fed for 24 hours the stock ration containing the feces marker. All definitely red feces were discarded. Filter paper was placed in the bottom of the cages to absorb urine; an attempt was made to separate hair from the feces.

In order to determine the amount of the amino acids which appeared in the feces as a result of metabolic processes, such as the excretion of enzymes into the digestive tract, the experimental period was followed by a standardizing period of 7 days in which a low nitrogen ration was fed. This ration contained 6% defatted whole egg to give a protein content of

approximately 4%. It has been shown by Mitchell and Carman ('26) and Bricker and Mitchell ('47) that whole egg protein when fed in these amounts is completely digested and utilized. The composition of all diets is given in table 1.

Food composition was measured and feces collections were made for the experimental and standardizing periods. In order to control scattering of food, the rations were fed in the

TABLE 1
Composition of rations

CONSTITUENTS	ROAST BEEF RATION	COTTONSEED FLOUR RATION	PEANUT FLOUR RATION	WHEAT RATION	EGG RATION
	%	%	%	%	%
Roast beef ¹	13.8		
Cottonseed flour ¹ (Proflo)		18.5	
Peanut flour ¹		..	16.4
Wheat ¹				64.5	..
Defatted whole egg ¹					6
Sucrose	10	10	10	10	10
Vitaminized starch ²	5	5	5	5	5
Starch	52.2	47.5	49.6	1.5	60
Wood pulp (alpha-flock B) ³	2	2	2	2	2
Butter fat	8	8	8	8	8
Cod liver oil	2	2	2	2	2
Salt mixture ⁴	4	4	4	4	4
NaCl	1	1	1	1	1
BaSO ₄	2	2	2	2	2

¹ Crude protein content of materials ($N \times 6.25$): Roast beef, 72.25; cottonseed flour, 54.07; peanut flour, 61.00; wheat, 15.54; egg, 69.88.

² Mitchell et al. ('45).

³ Brown Co., Berlin, New Hampshire.

⁴ Wesson ('32) NaF omitted.

form of a paste containing 10 ml of a dilute liver extract, prepared as indicated below, and 15 gm of the dry ration. The use of the liver extract was an important factor in maintaining satisfactory food consumption. Food residues were dried and weighed. Moisture determinations were made in order to adjust all food residue weights to the original moisture content of the prepared rations.

Preparation of liver extract

Five pounds of fresh beef liver were ground and then homogenized in the Waring Blendor with 6 liters of methanol. The protein was removed by centrifugation. The methanol was removed by distillation at reduced pressure and the residue dissolved in 5 liters of water and filtered. The nitrogen content was 0.58 mg per ml; the amino acid content was considered insignificant.

Preparation of roast beef

The roast beef sample was well done. Three pounds of boned prime rib were roasted for 2¼ hours at 350°F. The lean meat was ground in a food chopper and dehydrated at room temperature with the apparatus described by Lyman et al. ('46a). In order to facilitate thorough mixing of the ration, the meat was reground after drying.

Microbiological methods were used for the determination of the essential amino acids in the food and feces. Valine, leucine, and isoleucine were determined with *L. arabinosus* 17-5 (Kuiken et al., '43). *Streptococcus faecalis* (American Type Culture Collection no. 8043) was used for threonine, histidine (Lyman et al., '47) and tryptophane assays (Kuiken et al., '47), while *L. mesenteroides* P-60 was used to determine methionine (Lyman et al., '46b), arginine, lysine, and phenylalanine. The amino acid composition of the materials studied is given in table 2.

RESULTS AND DISCUSSION

Illustrative data for a single amino acid, lysine in roast beef, are given in table 3. The data show that the endogenous amino acid excretion was roughly proportional to the amount of food eaten. This is in accordance with the observations of Mitchell ('24) and Schneider ('35) on endogenous nitrogen excretion. The calculation of true amino acid availabilities was based on this concept. In general the correction to be applied to apparent availabilities was quite small.

TABLE 2

Amino acid composition of materials studied
(Expressed as percentage of the crude protein, N \times 6.25)

AMINO ACID	ROAST BEEF	COTTONSEED FLOUR	PEANUT FLOUR	WHEAT
Arginine	6.53	11.30	11.26	4.38
Histidine	3.27	2.57	2.16	2.51
Isoleucine	4.43	3.51	3.85	3.03
Leucine	8.77	6.23	6.74	6.82
Lysine	9.98	4.16	3.20	2.90
Methionine	3.13	1.61	1.02	1.35
Phenylalanine	4.25	5.20	5.08	5.15
Threonine	4.90	3.36	2.67	2.83
Tryptophane	1.30	1.46	1.20	1.35
Valine	6.03	4.84	4.64	4.50

TABLE 3

Metabolic data and the calculation of lysine availability in roast beef

RAT NUMBER	1	2	3	4	5
Metabolic data: roast beef ration, 9.97% protein					
Food intake (gm)	71.7	74.8	56.9	69.1	88.2
Lysine in food eaten (gm)	0.713	0.744	0.566	0.688	0.877
Weight of feces (gm)	4.487	4.432	3.506	4.877	5.452
Lysine in feces (%)	0.64	0.66	0.83	0.78	0.64
Total lysine in feces (mg)	28.7	29.2	29.1	38.0	34.9
Metabolic data: egg ration, 4.19% protein					
Food intake (gm)	62.6	65.0	45.3	46.0	69.2
Weight of feces (gm)	3.908	4.283	2.772	3.017	4.610
Lysine in feces (%)	0.66	0.66	0.66	0.66	0.63
Total lysine in feces (mg)	26	28	18	20	29
Lysine in feces per gram food eaten (mg)	0.42	0.43	0.40	0.43	0.42
Derived data					
Fecal lysine of metabolic origin (mg)	30.1	32.1	22.8	29.7	37.0
Fecal lysine of dietary origin (mg)	—1.4	—2.9	6.3	8.3	—2.1
Apparent lysine availability (%)	95.9	96.1	94.9	94.5	96.0
True lysine availability (%)	100.1	100.4	98.9	98.8	100.2

The essential amino acids in roast beef were found to be completely available to the rat under the described experimental conditions. As is shown in table 4 the true availabilities of these amino acids ranged from 99.2 to 100.7%. The total nitrogen of the meat protein was also found to be completely available. This result was to be expected in view of the finding of Mitchell et al. ('36) that the protein of beef round is completely digestible. It is of interest that roasting the beef did not decrease the availability of the amino acids.

TABLE 4
True availability of amino acids in roast beef

AMINO ACID	EXPERIMENT 1						EXPERIMENT 2
	Rat number					Average values	Average values
	1	2	3	4	5		
	%	%	%	%	%	%	%
Arginine	101.1	101.2	100.5	99.6	101.2	100.7	101.2
Histidine	101.3	100.8	100.0	99.6	100.7	100.5	100.2
Isoleucine	101.1	100.5	99.3	98.8	99.8	99.9	100.0
Leucine	100.3	100.0	99.6	98.5	100.3	99.7	99.9
Lysine	100.1	100.4	98.9	98.8	100.2	99.7	100.5
Methionine	100.0	100.9	99.4	99.5	100.7	100.1	100.7
Phenylalanine	99.7	99.7	98.3	97.6	98.7	98.8	100.8
Threonine	99.7	101.6	98.6	98.2	100.5	99.7	100.8
Tryptophane	99.0	100.2	98.5	97.9	100.3	99.2	100.1
Valine	99.8	100.2	99.1	97.6	100.2	99.4	100.2
Total nitrogen	100.4	101.6	99.7	99.1	99.7	100.1	

Quite different results were obtained with a sample of cottonseed flour². Marked variations in availability of individual amino acids were observed for this foodstuff (table 5). Although 93% of the arginine was available to the rat, only about 65% of the lysine was available. The values for the other amino acids ranged within these extremes. It will be noted that the agreement between values in the same experiment was satisfactory and that the average values were

² Proflco.

closely duplicated by a 2nd experiment in which a new set of animals was used.

In the case of peanut flour and wheat, a small loss of amino acids was found. In contrast with cottonseed flour, this loss was rather uniformly distributed among the various amino acids in the food. Availability values ranged from 94.8 to

TABLE 5
True availability of amino acids in cottonseed flour

AMINO ACID	EXPERIMENT 1					EXPERIMENT 2	
	Rat number					Average values	Average values
	1	2	3	4	5		
	%	%	%	%	%	%	%
Arginine	94.2	93.3	93.0	94.3	93.7	93.7	93.4
Histidine	90.5	88.0	87.2	89.7	89.3	88.9	89.9
Isoleucine	82.9	81.7	81.0	83.7	83.0	82.5	78.2
Leucine	83.9	80.4	78.6	81.6	79.5	78.8	75.4
Lysine	65.7	59.5	62.3	66.1	64.6	63.6	64.5
Methionine	82.3	79.8	77.3	82.4	81.7	80.7	81.6
Phenylalanine	87.1	85.8	85.6	88.0	86.7	86.6	88.6
Threonine	79.4	76.0	77.2	80.4	78.4	78.3	76.6
Tryptophane	87.7	87.7	90.4	90.0	90.7	89.3	90.8
Valine	81.4	79.1	77.0	74.1	81.8	76.7	78.6
Total nitrogen	88.3	87.5	86.7	88.1	87.5	87.6	

99.5% for peanut flour and from 92.8 to 98.8% for wheat (table 6).

Investigations of the type reported here are subject to the criticism that bacterial action in the lower intestine may have altered the distribution of amino acids in the feces. Complete evidence that errors due to this cause are quantitatively insignificant, will require further investigation. It appears unlikely that the high percentage of lysine found in the feces from the animals fed cottonseed flour was due to bacterial synthesis.

The results indicate that in certain cases information on the amino acid content of foodstuffs will be quite inadequate for evaluating the protein unless availability data are also ob-

tained. While close correlation was found between nitrogen and amino acid availabilities for roast beef, peanut meal and wheat, there were marked exceptions in the case of cottonseed meal. Further investigation will be necessary in order to determine whether such variations in individual amino acid availability in a single foodstuff are to be generally expected or whether cottonseed meal and soybean meal (Melnick et al., '46) are unusual exceptions.

TABLE 6
True availability of amino acids in peanut flour and wheat

AMINO ACID	TRUE AVAILABILITY ¹	
	Peanut flour	Wheat
	%	%
Arginine	99.5	96.4
Histidine	98.8	98.8
Isoleucine	97.2	95.0
Leucine	97.0	95.4
Lysine	97.0	92.8
Methionine	95.8	94.9
Phenylalanine	97.9	96.9
Threonine	94.8	92.2
Tryptophane	97.2	93.2
Valine	95.8	93.2
Total nitrogen	97.7	95.0

¹ Average values based on groups of 5 animals each.

It is probable that changes in the manufacturing procedure may influence the availability of amino acids in cottonseed products. All of the materials studied in this investigation were finely ground. The extent to which this may have influenced amino acid availability is not known.

SUMMARY

1. It was found that all 10 of the essential amino acids in roast beef are completely available to the rat.
2. Wide variations occurred in the availability of the individual amino acids in cottonseed flour. Lysine in this

protein source was only 65% available. The corresponding value for arginine in the same sample was 93%.

3. The availability of each of the essential amino acids in wheat and peanut flour was found to be relatively high. For wheat the values ranged from 92.2 to 98.8%, and for peanut flour from 94.8 to 99.5%.

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VITAMIN B₆, INOSITOL, AND NICOTINIC ACID IN THE NUTRITION OF THE TURKEY¹

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It is well established that turkey poult require vitamins A, D, E, and riboflavin, pyridoxine, pantothenic acid, nicotinic acid, choline, and vitamin B₆. They probably need vitamin K and they undoubtedly require thiamine, though so far as we are aware, no data on these points have been published, and it has been reported that they need inositol.

Richardson, Hogan and Kempster ('45) diluted a practical ration with a synthetic diet and observed that turkey poult which consumed it developed a spastic cervical paralysis. If the ration was not changed, death usually followed within 2 or 3 days. If pteroylglutamic acid was added to the diluted ration, the poult grew normally and, except for isolated cases of perosis, they were free from abnormalities. Jukes, Stokstad and Belt ('47), Russell, Taylor and Derby ('47) and Schweigert, German, Pearson and Sherwood ('48) confirmed the observations of Richardson, Hogan and Kempster. Jukes et al. noted that when the diet was deficient in pteroylglutamic acid, the erythrocytes were somewhat larger and more elongated than is normal. They estimated that the poult requires 80 μ g of the vitamin per 100 gm of ration. Russell, Taylor and Derby arrived at a much higher estimate, 200 μ g %. In

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1 group of 8 poults that survived on 150 μg % of pteroylglutamic acid, there were 4 cases of cervical paralysis. There was no paralysis in the group that received 200 μg %. Schweigert and coworkers reported that 80 μg % is insufficient for poults when the eggs from which they hatched were laid by hens that consumed a ration which contained only 42 μg % of the vitamin. Poults from this same group grew normally and had a low mortality rate when their diet contained 200 μg %. However, when the ration of the hens contained a liberal amount of pteroylglutamic acid, their poults responded as well when the diet contained 80 μg % of pteroylglutamic acid as when it contained 200 μg %. Three poults, from hens on their low PGA diet, developed cervical paralysis on a diet that contained 20 μg % of pteroylglutamic acid. None of the poults developed this symptom when their diet contained 80 μg % of the vitamin.

Briggs ('46) observed a high incidence of perosis in poults that received insufficient nicotinic acid and concluded that the minimum requirement is at least 3 to 5 mg per 100 gm of diet. His data indicated that the diet may have been improved by adding 10 mg of this vitamin to his basal diet. Jukes, Stokstad and Belt ('47) confirmed the report by Briggs, and noted no perosis in poults when the diets contained either 5 or 10 mg of niacinamide per 100 gm of food. Jukes et al. ('47) also reported that the poult requires inositol. When this substance was omitted from the diet, growth was retarded, a mild normocytic anemia developed, and some of the erythrocytes had abnormal nuclei. Patrick and associates ('43) reported that the incidence of perosis in poults was not reduced by including inositol in the diet and they apparently found no evidence that the poult requires that substance.

EXPERIMENTAL

The routine followed in the management of experimental turkey poults was described by Richardson, Hogan and Kempster ('45). Most of the poults were Standard Bronze, the others were Broadbreasted Bronze. The experimental periods

were 4 weeks in length and the weights of the poults and the incidence and degree of perosis were recorded at intervals of 1 week. A few of the poults were killed before the end of the experimental period either because they were crippled with perosis or because of infection. In most trials, the mortality rate was unduly high and seemed to have no relation

TABLE 1
Composition of the basal diet

	%		%
Casein	35	Wood pulp	3
Gelatin	10	Lard	10
Cerelose	37	Salts ¹	5
Vitamins ² per 100 gm of diet			
Vitamin A	2000 I.U.	Pyridoxine hydrochloride	1 mg
Vitamin D	290 AOAC U.	Ca-pantothenate	3 mg
Vitamin E	2.5 mg	Nicotinic acid	5 mg
2-methyl-1,4-naphthoquinone	2.5 mg	Choline chloride	400 mg
Thiamine hydrochloride	1 mg	Biotin	0.02 mg
Riboflavin	1 mg	Inositol	100 mg
Pteroylglutamic acid, ³ variable			

¹ Richardson and Hogan ('46).

² Vitamins A and D were purchased as a concentrate from Mead Johnson and Company, Evansville, Indiana. The pteroylglutamic acid was generously supplied by Dr. T. H. Jukes, Lederle Laboratories, Pearl River, New York. All other vitamins were generously supplied by Dr. D. F. Green of Merck and Company, Inc., Rahway, New Jersey.

³ Extinction coefficients of the sample we used, observed in this laboratory by Dr. B. L. O'Dell, indicated a purity of 95% according to the values of Bloom et al. ('44), and of 100% according to the values of Stokstad, Hutchings and Subbarow ('46).

to the adequacy of the diet. It was necessary to ship the poults in from some distance and it is believed that delays in transit, with exposure to cold and deprivation of food and water, had reduced the vitality of the weaker poults to a low ebb. The rations, described in table 1, were of the simplified type. The casein, prepared in the laboratory from skim milk, was precipitated with hydrochloric acid and extracted

thoroughly with dilute acetic acid. The composition of the basal diet was modified at times in minor respects in attempts at improvement, but since the modifications proved to be of no consequence they have been ignored and the data on similar diets have been combined. The basal diet most frequently used is described in table 1.

*The amount of pteroylglutamic acid required
by the turkey poult*

The growth rates reported in table 2 were highly variable but the weights were inferior when the rations contained less

TABLE 2

The amount of pteroylglutamic acid required by the turkey poult

GROUP ¹	1	2	3	4	5	6	7
Pteroylglutamic acid (mg/100 gm)	0.01	0.025	0.05	0.1	0.15	0.2	0.3-0.4
No. of separate trials	2	1	4	6	1	12	8
No. of poults	20	10	39	54	6	107	74
No. of poults killed ²	0	0	0	2	0	7	7
Mortality (%)	75	70	51	18	17	22	4
Hematocrit ³ reading (%)	32	35	35	33	36	38	36
Hemoglobin ³ in blood (%)	7.4	8.2	8.5	6.8	6.9	8.4	8.7
Perosis (%)	0	0	7.7	9	17	7.4	12
Perosis (score)	0	0	50	55	25	85	60
Cervical paralysis (%)	35	50	16	3.7	0	0	0
Broken feathers (%)	100	100	53	19	60	8	6
Broken feathers (score)	3.7	3.5	3.4	2	3	1.3	1
Avg. weight at 4 weeks (gm)	277	198	332	373	334	501	451

¹ As a rule the diets contained 100 mg % of inositol but the exceptions are not shown separately since they had no appreciable effect on the results.

² Perosis or diarrhea.

³ Taken at 3 weeks.

than 0.2 mg % of the vitamin. The poults grew as rapidly when the diet contained 0.2 mg % of the vitamin as when it contained larger amounts. The poults that received 0.2 mg % had an average weight of 501 gm at 4 weeks. We were unable to separate the sexes at that age, but presumably the

number of males and females was about the same. No attempt was made to search the literature for maximum growth rates, but the highest weight at 4 weeks recorded in our files was approximately 500 gm for males. Turkey poults grow rapidly on synthetic diets that are properly constructed.

When the diet contained less than 0.1 mg % of the vitamin, the mortality rate was increased, and of the 54 poults that received the diet which contained 0.1 mg %, 2 developed typical cervical paralysis. As the amount of the vitamin was reduced, the incidence of paralysis increased.

When the poults were 3 weeks old the hematocrit volume was determined by the method of Van Allen ('25). The blood for hemoglobin determinations was drawn from the ulnar vein and 0.05 ml was hemolyzed in 10 ml of distilled water. Two drops of concentrated ammonium hydroxide were added and the mixture was centrifuged for 20 minutes. Centrifugation was completed within 10 minutes after the ammonia had been added. A Pfaltz and Bauer protoelectric colorimeter, equipped with a 540 filter, was used for the hemoglobin determinations. With the aid of a standard response curve the deflection of the galvanometer was converted into percentage of hemoglobin in the blood.

The percentage of hemoglobin in the blood and the red cell volume were determined for each poult included in table 2, and the poults which received either 0.1 or 0.15 mg % of pteroylglutamic acid were mildly anemic. It was unexpected that these poults would be more anemic than were those that received still smaller amounts of vitamin B₁₂ and presumably this was due to the fact that the more susceptible poults in groups 1, 2, and 3 had died before the determinations were made. This difference was not reported by Russell, Taylor and Derby ('47), possibly because their observations were made when the poults were older. There was no correlation between the incidence of perosis and the amount of vitamin B₁₂ in the diet. The percentage of perosis shown in table 2 is the per cent of poults that developed the abnormality. The "degree of perosis" is an empirical rating of its severity. Mild

cases with swollen joints were marked 25. Moderate cases with markedly swollen joints and 1 foot turned to the side were marked 50. Severe cases in which the tendon of Achilles had slipped from the chondyle were marked 75. The most severe cases in which the poult had lost control of both legs were marked 100. It will be noted in table 2 that the incidence of perosis was not reduced when the amount of pteroylglutamic acid in the diet was raised to 0.3 mg %. Presumably this diet contained ample amounts of all recognized nutrients that are required to prevent perosis. The occurrence of the abnormality under these circumstances is regarded as evidence that the diet is still deficient in an unrecognized essential nutrient.

Several investigators have reported that the feathers are imperfect when chicks receive insufficient pteroylglutamic acid, and Russell, Taylor and Derby ('47) had the same experience with turkeys. Our observations on this point are described in table 2. The percentage of broken feathers is the percentage of poult affected. The score indicates the degree of damage. A score of 0 indicates perfect feathers and a score of 4 indicates the most extreme damage. It will be noted that when the diet contained less than 0.2 mg % of pteroylglutamic acid, the percentage of affected poult was high and the damage was extensive. When the diet contained 0.2 mg % or more of the vitamin, the condition of the feathers was vastly improved, but still they were not perfect. The close confinement may have contributed to the imperfections, but to us it seems probable that the synthetic diets were not entirely adequate and that a mild nutritional deficiency is the explanation of the minor defects in groups 6 and 7 of table 2.

Our data show that the minimum requirement of the turkey poult for pteroylglutamic acid is more than 0.15 mg % of the diet but not over 0.2 mg %. It may be, though, that the minimum requirement depends in some degree on the other constituents of the diet as was reported for chicks by Luckey, Moore, Elvehjem and Hart ('46). It is also possible that the minimum requirement depends in some degree on the previous history of the poult, as reported by Schweigert, German,

Pearson and Sherwood ('48). It seems reasonable, though, that the minimum allowance recommended for commercial practice should meet the maximum requirement.

The results of a more intensive study of the cellular constituents are summarized in table 3. The erythrocytes were counted by the method of Wiseman as described by Biester and Devries ('43, chap. 4, p. 69), and the leucocytes were counted by the method of Blain ('27-'28). The 2-slide method

TABLE 3
Observations on cellular constituents of the blood

GROUP	1	2	3	4 ¹
Pteroylglutamic acid (mg/100 gm)	0.10	0.15	0.20	
No. of poults in group	8	5	13	10
Arg. wt. of poults at 4 weeks (gm)	265	334	440	432
Hemoglobin in blood (%)	6.1	6.9	8.9	9.2
Hematocrit reading (%)	30	36	42	40
Erythrocytes, ² 10 ⁶ per mm ³	1.82	1.61	2.43	2.25
Leucocytes, 10 ³ per mm ³	12.8	17.2	20.6	24.8
Lymphocytes, 10 ³ per mm ³	5.965	10.303	9.085	11.855
Heterophils, 10 ³ per mm ³	6.016	5.710	10.332	11.507
Eosinophils, 10 ³ per mm ³	0.128	0.258	0.241	0.307
Basophils, 10 ³ per mm ³	0.371	0.516	0.812	0.794
Monocytes, 10 ³ per mm ³	0.294	0.396	0.127	0.248
Thrombocytes, 10 ³ per mm ³	18.230	17.450	33.400	36.680

¹ Practical control ration.

² All cellular constituents were counted on 5 poults in each group.

was used in making the smears for the differential white cell counts.² The blood was taken from the ulnar vein and the first drop was always discarded. The films were stained with Wilson's blood stain (200 mg of Wilson's stain dissolved in 50 ml of acetone-free methyl alcohol). After drying the blood film in the air, without previous fixation, the smear was covered with an excess of the stain. One and one-half minutes after the addition of the stain neutral distilled water was added, in a quantity equal to that of the staining fluid. The

² We are indebted to Dr. H. C. McDougale who developed the method and taught us how to use it.

mixture was allowed to stand for about 2 minutes (until a greenish film had formed on top of the staining mixture) and the preparation was washed with neutral distilled water for about 30 seconds, or until the thinner portions of the film became a bluish-purple in color. Schmeisser's method ('15) was used in making the classifications. Three hundred white blood cells were counted in each smear. Groups 2 and 3, which received 0.1 and 0.15 mg % of vitamin B₆, developed a mild macrocytic anemia. The erythrocytes also developed other abnormalities, including anisocytosis, poikilocytosis and mottled and abnormally shaped nuclei. The poults that received 0.2 mg % had about the same erythrocyte count, hematocrit reading and percentage of hemoglobin in the blood, as did the poults that consumed the practical control ration. When the ration contained 0.1 mg % of vitamin B₆ there was a marked reduction in the number of thrombocytes and leucocytes, including both lymphocytes and granulocytes. When the ration contained 0.15 mg % of vitamin B₆, the blood still had only one-half the normal number of thrombocytes, but the average number of leucocytes was only slightly depressed. Two of the 5 poults examined had a granulocyte deficiency. The leucocytes were also enlarged, with mottled and abnormally shaped nuclei. When the ration contained 0.2 mg % of pteroylglutamic acid, as in group 3, there was a slight but consistent decrease in the number of both thrombocytes and leucocytes. These counts leave the impression that even though the synthetic diets support an excellent rate of growth they are still slightly inadequate. There were some inconsistencies between groups, but presumably these could be explained by the variability of the poults at the time they reached the laboratory.

The requirement of the turkey poult for inositol

Our data on this point are shown in table 4 and give no definite indication that the amount of inositol in the diet has any influence on the weight, the number of red cells, the percentage of hemoglobin in the blood, or the differential cell

counts. However, when inositol was omitted from the diet, the leucocyte counts were lower than when it was added and the difference was statistically significant. Jukes and associates ('47) reported that when turkey poult received insufficient inositol they were mildly anemic and the erythrocytes were slightly abnormal. It is our view that the abnormality was due to a slight inadequacy in the supply of pteroylglutamic acid. Jukes et al. did not report leucocyte counts.

TABLE 4

The requirement of the turkey poult for inositol

GROUP ^{1,2}	1	2	3	4
Inositol (mg/100 gm)	100	0	100	0
No. of poults	10	6	7	7
No. of poults killed ³	0	0	2	0
No. of poults survived	8	6	5	5
Hematocrit reading (%)	43	43	41	40
Hemoglobin in blood (%)	9.2	9.6	8.6	8.4
Erythrocytes, ⁴ 10 ⁶ per mm ³	2.47	1.97	2.38	2.38
Leucocytes, ⁴ 10 ³ per mm ³	21.6	16.4	19.6	15.4
Thrombocytes, ⁴ 10 ³ per mm ³	32.02	25.66	34.78	34.28
Perosis (%)	30	17	0	14
Perosis (score)	75	25	0	50
Avg. weight at 4 weeks (gm)	452	408	428	489

¹ The basal ration contained 0.2-0.3 mg pteroylglutamic acid per 100 gm.

² Groups 3 and 4 were under observation at a later date than those in groups 1 and 2. They are shown separately in order to illustrate the variability in weight.

³ Perosis or diarrhea.

⁴ The cellular constituents were counted on 5 poults in each group.

The relation between nicotinic acid and the incidence of perosis

None of our diets contained less than 5 mg % of nicotinic acid, which is more than enough to prevent any specific symptoms of a deficiency. Briggs ('46) and Jukes and associates ('47) have presented evidence which indicates a relation between nicotinic acid and the incidence of perosis. Our data, presented in table 5, indicate that when the amount of nicotinic acid added to the basal synthetic diet was increased

from 5 to 10 mg % the incidence of perosis, and the severity of the abnormality, were decreased by about 50%. However, the syndrome was never completely prevented in poult which grew rapidly, which suggests that an unexplained factor is involved in the abnormality.

TABLE 5

The relation between nicotinic acid and the incidence of perosis

GROUP ¹	1	2
Nicotinic acid (mg/100 gm)	5	10
No. of separate trials	6	6
No. of poult	57	53
No. of poult killed ²	4	2
Mortality (%)	12	7
Hematocrit reading (%)	37	37
Hemoglobin in blood (%)	8.5	8.7
Perosis (%)	19.3	7.3
Perosis (score)	87	58
Avg. weight at 4 weeks (gm)	445	467

¹ The basal ration contained 0.2-0.3 mg pteroylglutamic acid per 100 gm.

² Perosis or diarrhea.

SUMMARY AND CONCLUSIONS

1. The amount of pteroylglutamic acid required by the turkey poult for maximum growth and normal development is approximately 0.2 mg %.

2. Under our experimental conditions the addition of inositol had no appreciable effect on either growth or the differential leucocyte count. When inositol was omitted from the diet the number of leucocytes was subnormal.

3. The incidence of perosis in turkey poult was decreased by increasing the nicotinic acid content of the diet from 5 to 10 mg %. Perosis was never completely absent in spite of the fact that the rations contained all factors known at present to prevent it.

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THE VITAMIN A CONTENT OF HUMAN BLOOD PLASMA AS AN INDEX OF CAROTENE UTILIZATION ¹

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TWO FIGURES

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The biological activity of the carotenoid pigments apparently depends upon the ability of the animal body to convert these pigments into vitamin A. Guggenheim ('44), Fraps and Meinke ('45) and Kemmerer and Fraps ('45) in experiments with rats have shown that the carotenes from different food sources are not equally well utilized. In a series of experiments with human subjects, Booher, Callison and Hewston ('39) and Booher and Callison ('39) have also shown that carotenes from various sources have different biological values. Their investigations also demonstrated that the human subject is less efficient than the rat in the utilization of carotene as a source of vitamin A. It appears, therefore, that neither chemical analysis nor biological assay by the rat-growth method of foods containing carotene can be depended upon as a measure of the vitamin A value for human beings.

The investigation of the utilization of carotenes is made difficult, as compared with the water-soluble vitamins, by the fact that vitamin A is stored in the liver in relatively large

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amounts and may be retained for long periods of time. Subjects must, therefore, be depleted of their reserve stores of vitamin A before any experimental work can be carried out. An increase in the dark adaptation time (night blindness) has generally been used as the criterion for determining the point at which the reserve vitamin A has been exhausted. The dark adaptation time has also been used for measuring the response to vitamin A supplementation. This method has, however, been criticized on the basis of non-specificity. Also the studies of Bodansky, Lewis and Haig ('41) and Lewis, Bodansky, Falk and McGuire ('42) provide data which lead to the conclusion that the level of vitamin A in the blood plasma may be a more sensitive index of vitamin A-status than the dark adaptation time.

The following study was carried out in an effort to determine the feasibility of using the level of vitamin A in the blood plasma as a measure of the utilization of carotenoids by humans.

EXPERIMENTAL

The general plan of the experiment was to place a human subject on a vitamin A-low diet until the level of vitamin A in the blood plasma dropped significantly. The individual would be standardized by determining the changes in the blood level resulting from the administration of known amounts of vitamin A. The subject could then be used to measure the efficiency of the utilization of carotenoids by measuring the level of vitamin A in the blood after the administration of known amounts of the test food. The subject studied was a female, 34 years old and in good general health.

Experimental periods

The experiment which lasted over 2 years was divided into 3 periods.

1. *Preliminary period.* This lasted for 49 days, with the subject on a normal diet, during which the carotene and vitamin A of the blood were determined at frequent intervals in order to have a measure of the normal levels for this subject.

2. *Depletion period.* The subject received a vitamin A-low diet in order to exhaust the reserve supply of vitamin A. This period covered 598 days, but included 2 rather extended periods of interruption. The time was divided as follows: depletion — 166 days; interruption — 72 days; depletion — 81 days; interruption — 164 days; depletion — 115 days. The total number of days actually on the depletion diet was 362. The periods of interruption were not planned as part of the experiment, but were the result of unexpected circumstances which caused the subject to be away from the laboratory for these periods.

3. *Experimental period.* This was 112 days, during which the subject remained on the depletion diet, but received supplements of vitamin A or papaya.

Diet

The vitamin A-low diet, which was estimated to contain 100–200 I.U. vitamin A per day, was similar to that of Booher et al. ('39). A vitamin capsule containing vitamins of the B-complex and ascorbic acid was taken daily and an iron tablet was taken 3 times each week. This diet is referred to as the basal diet.

During periods when the experiment was interrupted, the subject ate a normal diet except that foods unusually rich in vitamin A activity such as liver, carrots and spinach were avoided. This diet is referred to as the normal-restricted diet.

No attempt was made to regulate the dietary intake of vitamin E and no supplements of this vitamin were taken during the depletion period. During the periods of vitamin A supplementation, 100 mg of a vitamin E concentrate³ containing 34% natural mixed tocopherols were administered with the vitamin A supplement. Thus the possibility of variation in the utilization of the vitamin A of the supplements due to the influence of vitamin E was removed, since this factor was always present in adequate amounts.

³ Distillation Products, Inc., Rochester, N.Y.

Determination of blood levels

The levels of vitamin A and carotene in the blood were determined at frequent intervals during the preliminary period, at least once a week during the depletion period, and at least twice a week during the experimental period. The blood samples, which were taken in the morning, were analyzed for both vitamin A and carotene according to the method of Kimble ('39) using 5 ml of plasma. The factors used to convert the L values obtained with the Evelyn colorimeter to International Units of vitamin A and μg of carotene and also the factor for correcting L-620 for the presence of carotene were determined in this laboratory. For the determination of the carotene factor, a sample of crystalline carotene containing 90% β -carotene and 10% α -carotene⁴ was used. A sample of distilled vitamin A esters⁵ which had been especially prepared for use as a standard was used to determine the factor for vitamin A.

Supplements

The supplements of vitamin A were administered in the form of a distilled vitamin A concentrate suitably diluted with cottonseed oil. The original concentrate and the diluted samples, which were freshly prepared each week, were assayed chemically by means of the antimony trichloride reaction.

The papaya supplements were given in the form of a frozen puree prepared with the aid of a Waring Blender. A large volume was well-mixed and then placed in half-pint packages and quick-frozen. Two separate lots were prepared and held in a freezing locker until used. The carotene content of each of these lots was determined colorimetrically with the Evelyn colorimeter. Samples of frozen papaya were extracted according to the procedure of Moore and Ely ('41). The extracts were saponified with alcoholic KOH at room temperature and the carotenes were transferred to petroleum

⁴ S. M. A. Carotene.

⁵ Distillation Products, Inc., Rochester, N. Y.

ether. The washed and dried extracts were chromatographed on Hyflo Super Cel (Wilkes, '46) and separate determinations were made of β -carotene and cryptoxanthin, the 2 biologically active carotenoids found to be present in the papaya. The values obtained were converted to International Units of vitamin A on the basis that $0.6\text{ }\mu\text{g}$ β -carotene or $1.2\text{ }\mu\text{g}$ cryptoxanthin equals 1.0 I.U. of vitamin A activity. The average values for the 2 samples were 1230 and 1500 I.U. per 100 gm, of which about 80% was contributed by cryptoxanthin.

RESULTS AND DISCUSSION

The changes in the levels of vitamin A and carotene in the blood which occurred during the depletion period are shown in figure 1. The average and range of 9 values determined during the 49 days of the preliminary period were 133 ± 10 I.U. vitamin A and $149 \pm 25\text{ }\mu\text{g}$ carotene per 100 ml of plasma.

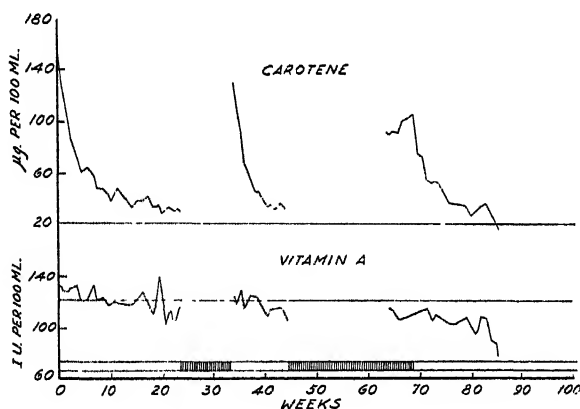


Fig. 1 The levels of vitamin A and carotene in the blood during the depletion period. The bar along the bottom of the chart indicates the type of basal diet: open spaces—vitamin A deficient diet; lined spaces—more liberal diet, but containing no foods very rich in vitamin A.

The change from a normal diet to the vitamin A-low diet resulted in an immediate and sharp drop in the carotene level of the blood. This occurred, as expected, each of the 3 times that the change was made from a normal to the vitamin A-

low diet. The vitamin A level, on the other hand, showed no consistent change during the first 140 days, but maintained a level between 118 and 140 I.U. per 100 ml. After the first 140 days a slight drop occurred and the level fluctuated between 102 and 117 I.U. per 100 ml for the next 24 days. At this time, however, the experiment was interrupted and the subject changed to the normal-restricted diet. After 72 days the subject returned to the basal diet for 81 days during which there was a slight but definite drop, as shown on figure 1, from about 120 I.U. vitamin A per 100 ml to about 110 I.U. per 100 ml. After a second interruption (164 days), the subject returned to the basal diet with a blood plasma vitamin A level of about 115 I.U. per 100 ml. The level then dropped gradually over a period of 106 days. At the end of this time a relatively sharp drop occurred to a level below 90 I.U. vitamin A per 100 ml of plasma. Nine days later the blood level was 76 I.U. per 100 ml — about 60% of the normal value for this subject. The subject was, therefore, considered to be sufficiently depleted and supplements were begun. There were no other symptoms or evidences of vitamin A depletion such as loss of weight, roughness of the skin or subjective night blindness.

Graded doses of vitamin A esters in oil were given in order to determine the amount of vitamin A required per day to maintain a blood level only slightly below normal. The object was to meet the daily needs of the body but to prevent the accumulation of reserve supplies in the liver so that the level in the blood would be dependent upon the intake. The amounts given per day and the blood levels found are shown in figure 2. The dosage was gradually increased from 2000 I.U. to 3500 I.U. per day over a period of 28 days, during which there was first a leveling off and then an increase in the level of vitamin A in the blood. At the end of 32 days, however, there occurred an unexpected drop in the blood level. The dosage was, therefore, raised to 10,000 I.U. per day and continued at that level for 7 days. During that time the subject developed a cold with a temperature of 100°F. She was then

given a more liberal diet plus supplements of from 10,000 to 100,000 I.U. vitamin A per day for 3 days. But, in spite of these high doses of vitamin A, the blood level continued to fall and reached the low level of 46 I.U. per 100 ml at the end of the 3 days. At this point the subject was given 100,000 I.U. vitamin A plus about 200 gm of cooked liver. This resulted in an increase in the plasma vitamin A to a value of 126 I.U. per 100 ml on the following day.

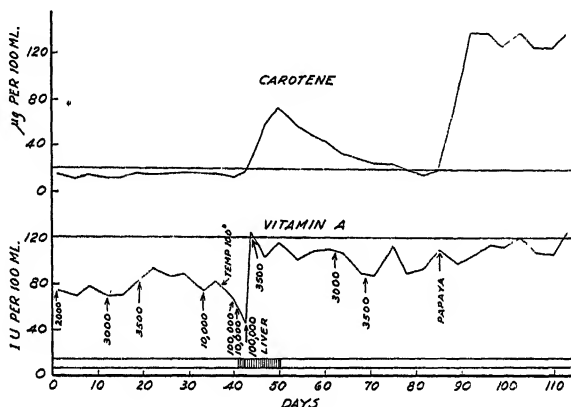


Fig. 2 The levels of vitamin A and carotene in the blood during the experimental period. The numbers with the arrows indicate I.U. of vitamin A administered per day. The bar along the bottom of the chart indicates the type of basal diet: open spaces — vitamin A deficient diet; lined spaces — more liberal diet.

For the next 6 days the subject continued to receive a more liberal diet plus 3500 I.U. per day. During this time the plasma level was maintained at approximately 110 I.U. vitamin A per 100 ml. The experiment was, therefore, resumed and the subject was returned to the basal diet plus a supplement of 3500 I.U. vitamin A per day. This dosage was continued for 11 days during which the plasma level averaged 111 I.U. per 100 ml with a range of 103 to 118. The dosage was then reduced to 3000 I.U. per day for 8 days and the blood level dropped to 90 I.U. per 100 ml. The level gradually rose again to about 100 I.U. per 100 ml when the dosage was restored to 3500 I.U. per day for 16 days. It was concluded from these

data that 3500 I.U. vitamin A per day were required by this subject in order to maintain the vitamin A in the plasma at a level of 100 to 110 I.U. per 100 ml.

Supplements of papaya were then given in amounts calculated, on the basis of the chemical assays, to provide 3500 I.U. of vitamin A activity per day. These supplements were fed over a period of 28 days during which the average level of vitamin A in the plasma rose slightly and averaged 112 I.U. per 100 ml. Thus the supplements of papaya were just as effective in maintaining the level of vitamin A in the blood as the supplements of vitamin A esters in oil — perhaps even more so since the trend was rather consistently upward during this period. This interpretation is, however, based on the assumption that the chemical analysis of the papaya samples quantitatively measured all the pro-vitamin carotenoids present. Since cryptoxanthin contributed over 80% of the calculated vitamin A activity of these samples, it may also be concluded that cryptoxanthin was well-utilized by this subject.

This experiment was unnecessarily long because of the interruptions during the depletion period and because of the unexpected drop in the plasma level which occurred in the middle of the experimental period, probably as the result of the subject's catching a cold. Clausen ('43) and Brenner and Roberts ('43) have reported that marked decreases occur in the level of vitamin A in the blood of subjects with infections or fever. Also this subject was probably depleted more than was necessary. The experimental period probably could have been started at the end of the first 166 days.

This experiment has demonstrated that the blood level can be used as an index for the study of the utilization of carotenoids. The level of vitamin A in the plasma of a depleted subject was shown to increase or decrease as the amount of vitamin A or carotene ingested increased or decreased. The data obtained, however, do not indicate that the blood plasma level is more sensitive than the dark adaptation time as an index of vitamin A nutrition although it is undoubtedly a more specific measure.

SUMMARY AND CONCLUSIONS

A single subject was placed on a vitamin A-low diet for 3 periods of 166, 80 and 115 days, respectively. Between these 3 periods were 2 periods of moderate vitamin A intake of 73 and 163 days, respectively.

The level of carotene in the plasma dropped rapidly each time the change was made to the vitamin A-low diet. The vitamin A level, on the other hand, did not show any significant change for 140 days, then dropped very gradually over a long period of time and finally made a rather abrupt drop. This drop was taken to indicate the end of the depletion period. The plasma levels were 149 μ g carotene and 133 I.U. vitamin A per 100 ml when the experiments began and 15 μ g carotene and 76 I.U. vitamin A per 100 ml at the end of the depletion period.

The feeding of increasing amounts of vitamin A (distilled esters in oil) resulted in a gradual increase in the vitamin A level in the plasma. An intake of 3500 I.U. per day maintained a level of 100 to 110 I.U. per 100 ml in the plasma and was taken to be a satisfactory intake for maintenance for this subject. Amounts of papaya which, according to chemical assay, provided 3500 I.U. of vitamin A activity per day in the form of carotenoids were then fed for 28 days. The plasma level of vitamin A was maintained between 100 and 120 during this period showing that the carotenoids of the papaya were well-utilized by this subject. Cryptoxanthin contributed 80% of the estimated vitamin A activity of the papaya samples.

These results show that the plasma level of vitamin A can be used as a measure of the utilization of carotenoids by human beings. The data obtained, however, do not indicate that the blood plasma level is more sensitive than the dark adaptation time as an index of vitamin A nutrition, although it is undoubtedly a more specific measure.

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THE EFFECTS OF SOYA LECITHIN ON THE ABSORPTION, UTILIZATION AND STORAGE OF VITAMIN A AND CAROTENE IN THE WHITE RAT

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INTRODUCTION

It has been reported by several investigators (McCoord and Luce-Clausen, '34; Davies and Moore, '34; Bauman et al., '34; LePage and Pett, '41) that the percentage utilization of dietary vitamin A and carotene is small, due to poor absorption and considerable destruction. Hickman et al. ('42), Sherman ('42), and others have demonstrated that the destruction of these constituents can be partially inhibited by the simultaneous ingestion of alpha-tocopherol and other fat soluble antioxidants that are normally present in certain fats and oils. Adlersberg and Sobotka ('43a) reported that in sprue the ingestion of vitamin A did not result in an increase in the blood level, but when lecithin was given at the same time there was a moderate rise in the serum vitamin A. In normal individuals, they (Adlersberg and Sobotka, '43b) found a rise of 41% in the blood 4 hours after a test dose of 18,000 I.U. of vitamin A. When lecithin was fed in addition, the rise in blood vitamin A was of the order of 200%. Slanetz and Scharf ('43, '45) observed a greater growth response when lecithin was fed to vitamin A-depleted rats along with vitamin A and carotene. They demonstrated also that lecithin mark-

¹ Graduate Fellow supported by the Government of India.

edly influenced both liver storage and blood levels of vitamin A. These authors have postulated that lecithin contains an unknown factor which influences carotene and vitamin A utilization in the rat.

In a previous contribution from this laboratory (Esh et al., '47), it was observed that when lecithin was fed to cows along with vitamin A during the later stages of the gestation period, more vitamin A was transferred to the colostrum milk as well as to the newborn calves by way of the placenta. Moreover, when a mixture of lecithin and vitamin A was added to a skim milk ration of newborn calves, they grew quite well and the blood vitamin A and liver storage were comparable to those of colostrum-fed calves.

In the investigation reported herein, a study was made in rats of the effect of feeding lecithin during the vitamin A depletion period and during the vitamin A and carotene supplementation periods on the rate of growth, plasma vitamin A level and liver storage of vitamin A.

EXPERIMENTAL

Experiment I. Soya lecithin and vitamin A

Fifty young albino rats reared under comparable conditions in our breeding colony were allotted to this experiment at weaning (24 days of age). Their weights varied from 48 to 52 gm. They were divided into 2 groups of equal sex and litter distribution and depleted of vitamin A on rations (a) and (b) (see table 1).

The rations were freshly prepared each week and stored in a refrigerator. The rats were maintained in individual cages and the rations provided ad libitum for 39 days. Weights were recorded weekly for the first 3 weeks and twice weekly thereafter. The same feeding and management routine was followed in each of the 3 experiments. Four rats were sacrificed from each group at the end of 3 weeks and a second set of 4 from each group at the end of the depletion period (39 days). The blood from each group of 4 animals was pooled

for serum vitamin A analysis and the livers obtained for individual liver vitamin A storage determination.

At the end of the depletion period the animals were regrouped as shown in table 2, experiment I. This regrouping was done to determine whether there was a carry-over effect of the depletion diet to the period of vitamin A supplementation. At this point vitamin A supplementation began.

TABLE 1
Percentage composition of the basal rations used

INGREDIENT	R A T I O N				
	a	b	c	d	e
Corn starch	50	50	65	65	65
Commercial dextrose (Cerelese)	17	17			
Casein, vitamin free	18	18	18	18	18
Hydrogenated vegetable fat (Crisco)	10	8	12	10	11.93
Crude soya lecithin ¹	..	2		2	
Choline chloride					0.07
Salt mixture ²	4	4	4	4	4
Yeast extract ³	1	1	1	1	1
Distilled water	Ad libitum				

¹ "Centrol II." Lecithin from Central Soya Company, Inc., containing 65% phosphatides suspended in 35% soybean oil. The phosphatides consist of approximately 50% lecithin and 50% cephalin.

² U.S.P. XII mixture no. 2 for vitamin A-free ration, to which were added traces of KI and CuSO₄.

³ Yeast extract supplied through the courtesy of Mead Johnson and Co., Evansville, Indiana.

A concentrate of the natural vitamin A ester was diluted with cottonseed oil and 5 µg of the vitamin administered to each rat daily. The vitamin A feeding was continued for 21 days. The rats were then sacrificed, the blood from each group pooled for serum vitamin A determination, and the livers obtained for individual liver vitamin A storage estimation.

Experiment II. Soya lecithin, choline and vitamin A

Since choline is a physiologically important group in the lecithin molecule, the results obtained in experiment I

prompted an investigation of the effect of choline on the absorption and liver storage of vitamin A. Fifty-four weanling albino rats from the same breeding colony, and raised under similar conditions, were divided into 3 groups of equal sex and litter distribution and depleted of vitamin A on rations (c), (d), and (e) (see table 1). The amount of choline included

TABLE 2

Distribution of the experimental animals in the various dietary groups of the 3 experiments

EXPERIMENT I			EXPERIMENT II			EXPERIMENT III		
Total animals	Depletion ¹	Repletion	Total animals	Depletion	Repletion	Total animals	Repletion	Depletion
50	25,a ²	8,a	54	18,c	6,c	42	21,c	7,c
		8,b			6,d			7,d
	25,b ²	8,a		18,d	6,c		21,d	7,c
		8,b			6,d			7,d
					6,e			7,e
					6,c			7,c
					6,d			7,d
					6,e			7,e

¹ The letter following the number indicates the ration (see table 1).

² Four animals from each of these groups were sacrificed at the end of 3 weeks and 4 more at the end of the depletion period. Two died.

in ration (e) was that calculated to be in the lecithin added to ration (d). This amount is substantially greater than that fed by Slanetz and Scharf ('45).

At the end of the depletion period (39 days) each original group was again divided into 3 groups as indicated in table 2 and 5 µg of vitamin A ester administered daily for the next 21

days. At the end of the 21-day repletion period the animals were exsanguinated and the livers obtained for vitamin A assay.

Experiment III. Soya lecithin, choline and carotene

This experiment was undertaken in an attempt to verify the unexpected effect of choline feeding and to study the effect of choline, lecithin and carotene feeding on the liver storage of vitamin A.

Forty-two weanling albino rats were divided into 2 comparable groups and depleted of vitamin A on rations (c) and (d) (see table 1). At the end of the depletion period (39 days) the animals were allotted to 6 sub-groups as indicated in table 2, experiment III. For the next 21 days each rat received a daily dose of 20 μ g of β -carotene in cottonseed oil solution. Twenty-four hours after the last carotene feeding the animals were exsanguinated and the livers removed for assay.

Assay-procedure

Blood serum vitamin A was determined by the method of Kimble ('39). Vitamin A was determined on the entire liver by the method of Lewis et al. ('42) with minor modifications.

RESULTS

Experiment I

The rats in the lecithin group made more rapid and greater weight gains during the depletion period. Weights of the animals on the basal vitamin A-free ration remained almost constant after 2 weeks, while the lecithin-fed group continued to gain until near the end of the depletion period. During the second week there was a decrease in the weight of both groups. The cause of this unexpected change is not certain. It is thought to have resulted from the extremely hot weather during the week.

Three rats in the lecithin group showed symptoms of vitamin A deficiency after 4 weeks. Toward the end of the deple-

tion period many of the rats of both groups showed the classic symptoms of avitaminosis A. Two of them died after 35 days.

The results of the vitamin A analyses of the blood sera and livers of the rats sacrificed at the end of 3 weeks and at the end of the depletion period are presented in table 3. It will be noted that the liver reserves were low at the end of 3 weeks and essentially exhausted at the end of the depletion period. Although the data are limited, the results indicate that the liver reserves are exhausted more rapidly when lecithin is fed and this is accompanied by lower blood serum vitamin A values.

During the vitamin A supplement feeding period the rats which received lecithin made greater weight gains, as shown in table 4. The liver storage of vitamin A was also greater in the lecithin-fed group. A statistical analysis of the liver storage data showed significance at the 1% level.

There appears to be a slight carry-over effect of lecithin feeding during the depletion period but this effect was not found to be statistically significant. The serum vitamin A at the end of the repletion period was found to be slightly lower when lecithin was fed. These blood data are too few for statistical analysis. Similar results were obtained in experiment III, however, as shown in table 7.

A preliminary inspection of the data indicated a sex difference in liver storage of vitamin A. This was verified by a statistical analysis of the data for the sexes presented in table 8.

Experiment II

The results of this experiment are summarized in table 5. During the depletion period both the lecithin- and choline-fed groups made greater weight gains than the control (basal) group. An analysis of these data showed the difference between the gains of the lecithin group and the control group to be significant at the 1% level. The difference between the choline group and the control group was not significant.

As in the first experiment, the liver storage of vitamin A was significantly higher (1% level) when lecithin was fed with vitamin A. The liver storage in the choline-fed rats was not significantly different from that in the control group.

TABLE 3

The effect on blood and liver vitamin A of lecithin feeding during vitamin A depletion

DEPLETION RATION	AT THE END OF 3 WEEKS			AT THE END OF THE DEPLETION PERIOD		
	No. of rats	Serum A	Total liver A	No. of rats	Serum A	Total liver A
		$\mu\text{g}/100\text{ ml}$	μg		$\mu\text{g}/100\text{ ml}$	μg
Basal	4	18	1.1	4	15	0.5
Basal + lecithin	4	16	0.6	4	13	0.3

TABLE 4

Effects of lecithin on the rate of growth and liver storage of vitamin A during the feeding of vitamin A (5 μg daily) for 21 days (8 rats in each dietary group)

SUPPLEMENT IN ADDITION TO VITAMIN A	GAIN IN WEIGHT (gm)			VITAMIN A ($\mu\text{G/LIVER}$)			PERCENTAGE OF VITAMIN A STORED
	Depletion diet			Depletion diet			
	(a)	(b)	Av.	(a)	(b)	Av.	
Lecithin	32.9	33.2	33.0	20.2	22.8	21.5	20.5
None	28.0	32.0	30.0	16.4	18.2	17.3	16.5

TABLE 5

Effects of lecithin and choline on the rate of growth and liver storage of vitamin A during the feeding of vitamin A (5 μg) daily for 21 days (6 rats in each dietary group)

SUPPLEMENT IN ADDITION TO VITAMIN A	GAIN IN WEIGHT (GM)				VITAMIN A (μ G/LIVER)				PER- CENTAGE OF VITA- MIN A STORED
	Depletion diet ¹				Depletion diet ¹				
	(c)	(d)	(e)	Av.	(c)	(d)	(e)	Av.	
Lecithin	33.0	36.0	27.0	32.0	17.0	21.2	18.0	18.7	17.9
Choline	29.0	27.3	22.0	26.1	14.0	12.0	12.7	12.9	12.3
None	26.0	27.0	25.0	26.0	12.2	14.8	13.1	13.3	12.6

¹ See table 1 for description of depletion diets.

Experiment III

The weight gains during the depletion period were so similar to those obtained in experiment I, and in the groups fed the basal ration and the basal ration plus lecithin of experiment II, that the data are not reported. Again the lecithin basal group made significantly greater (1% level) gains. The

TABLE 6

Comparative effects of lecithin and choline on the rate of growth and liver storage of vitamin A and "carotene" during the feeding of carotene (20 μ g daily) for 21 days (6 rats in each dietary group)

SUPPLEMENT IN ADDITION TO CAROTENE	GAIN IN WEIGHT (GM)			VITAMIN A (μG/LIVER)			"CAROTENE" (μG/LIVER)			PER- CENTAGE OF VITA- MIN A STORED
	Depletion diet			Depletion diet			Depletion diet			
	(c)	(d)	Av.	(c)	(d)	Av.	(c)	(d)	Av.	
Lecithin	25.6	26.2	25.9	65.5	67.5	66.5	3.7	2.6	3.1	32.4
Choline	22.0	24.0	23.0	52.9	58.4	55.6	3.7	2.6	3.1	27.2
None	14.3	20.0	17.0	51.7	59.0	55.3	2.7	1.9	2.3	26.8

TABLE 7

Comparative effects of lecithin and choline on blood serum vitamin A following the feeding of vitamin A (5 μ g daily) or carotene (20 μ g daily) for 21 days

SUPPLEMENT IN ADDITION TO VITAMIN A OR CAROTENE	SERUM VITAMIN A (μ G/100 ML)					
	Vitamin A supplemented groups (8 rats in each group)			Carotene supplemented groups (6 rats in each group)		
	Depletion diets			Depletion diets		
	(c)	(d)	Av.	(c)	(d)	Av.
Lecithin	32.9	37.4	35.1	42.5	37.3	39.9
Choline	45.2	42.3	43.7
None	36.5	41.5	39.0	45.2	39.9	42.5

results of the carotene supplementation are summarized in tables 6 and 7. When either lecithin or choline was fed with the carotene, the weight gains were greater (1% level of significance) than when carotene was fed with the basal diet alone. There was no significant carry-over effect of the depletion diets. The lecithin-supplemented group stored more

vitamin A (significant at the 1% level) than either of the other 2 groups, and choline produced no effect in this respect.

It will be noted that small amounts of pigment estimated as "carotene" were found in the livers following carotene feeding. Both "carotene" and vitamin A were converted to International Units (micrograms carotene \times 1.66 plus micrograms vitamin A \times 3.33) and the percentage storage calculated. While the general pattern of storage on the various diets was similar to that when vitamin A was fed (experiments I and II), a higher percentage storage was obtained when 20 μ g of carotene were fed.

TABLE 8

Influence of sex on the liver storage of vitamin A in rats fed 5 μ g of vitamin A or 20 μ g of carotene daily

SUPPLEMENT IN ADDITION TO VITAMIN A OR CAROTENE	NO. OF RATS	AVERAGE LIVER STORAGE (μG)		DIFFERENCE BETWEEN ♂ AND ♀ OF SAME GROUP	DIFFERENCE BETWEEN ♂ OF LECITHIN AND NON- LECITHIN GROUP	DIFFERENCE BETWEEN ♀ OF LECITHIN AND NON- LECITHIN GROUP
		♂	♀			
Vitamin A-fed groups						
None	16	14.6	20.7	41.6 ¹
Lecithin	16	19.4	24.3	25.1 ²	32.5 ¹	17.1 ²
Carotene-fed groups						
None	12	45.4	65.3	43.8 ¹
Choline	12	44.6	66.8	49.8 ¹
Lecithin	12	56.8	76.5	34.7 ¹	25.1 ²	17.2 ²

¹ Significant at the 1% level (probability of happening by chance 1 in 100).

² Significant at the 5% level (probability of happening by chance 1 in 20).

³ Not significant.

As in the previous experiments, a sex difference in vitamin A storage was evident. The liver storage data are summarized by sex in table 8. The sex difference in liver vitamin A storage was highly significant, regardless of the supplement fed in addition to carotene.

Again, as in the previous experiments, the blood plasma vitamin A was lower in the group which received both lecithin and carotene.

DISCUSSION

Several investigators (Brenner et al., '42; Davies and Moore, '35; Horton et al., '41; Popper and Greenberg, '41) have observed the rapid loss of vitamin A reserves in rats fed a vitamin A-free diet. The feeding of lecithin appears to hasten the decline in liver stores, but the superior growth response indicates either that the loss of the vitamin by destructive breakdown is retarded, or that greater economy is effected in its functional metabolic processes.

Adlersberg and Sobotka ('43b) and Augur et al ('47) have shown that lecithin enhances the digestion and absorption of fat, and Muelder and Kelly ('42) have produced evidence that a minimum dietary fat level is necessary for the optimum absorption and utilization of vitamin A, which in turn is responsible for greater weight gains. In the experiments reported herein no vitamin A was fed during the depletion period, and the enhanced growth rate accompanied by lower levels of vitamin A in both the blood and liver which resulted from the addition of lecithin to the depletion diet indicates a primary effect on the mobilization and utilization of the vitamin A reserves.

The greater growth response and liver storage of vitamin A in the rats receiving both lecithin and vitamin A indicate clearly both increased absorption and more effective utilization. The slight carry-over effect of lecithin feeding during the depletion period in increasing growth and liver storage during the vitamin A repletion period also supports this view.

Greenberg and Popper ('41) and Brenner et al. ('42) demonstrated the presence of sufficient vitamin A in the retina and blood even in the absence of liver stores. This probably explains the continuation of growth toward the end of the depletion period and indicates that lecithin may have aided in the mobilization and effective utilization of the vitamin from body depots other than the liver. The fact that lower blood levels of vitamin A were observed in the animals being fed lecithin, even though they gained more weight and stored

more of the vitamin in the liver, is further evidence that lecithin aids in vitamin A utilization.

The results of these experiments support the observations that lecithin feeding increases liver vitamin A storage, as reported by Slanetz and Scharf ('45), and conserves the hepatic reserves of this vitamin, as reported by Polskin ('40).

Crude soya lecithin contains a number of compounds that might be suspected of being responsible for the observations on growth and liver storage of vitamin A. Scharf and Slanetz ('44) and Patrick and Morgan ('44) presented evidence that α -tocopherol was not the active principle responsible for these effects. Since choline plays an important role in fat metabolism, we considered it of interest to compare its effects with those of lecithin under similar experimental conditions.

Choline feeding did not result in greater weight gains during the depletion period or in greater weight gains or increased liver storage of the vitamin during the vitamin A repletion period. The significant carry-over effect of choline feeding during the depletion period in decreasing the growth response to vitamin A feeding is difficult to explain. Thorbjarnarson and Drummond ('38) observed that vitamin A depletion could be accelerated by causing a partial dispersal of fat from the liver by the administration of choline. This effect was not evident from our experiments. Our choline-fed rats actually grew as well as the control group during the depletion period and they did not show deficiency symptoms at an earlier date.

It is possible that the lecithin effect is due to the fatty acids of lecithin, since it has been shown by Sherman ('40, '42) that some unsaturated fatty acids aid in the absorption and metabolism of vitamin A. A more probable explanation is that the lecithin molecule acts as an intact unit, or that the crude lecithin used contains some compound other than lecithin, choline or α -tocopherol which is the active principle.

The greater liver storage of vitamin A observed when carotene was fed in these experiments suggests the possibility

that, up to a certain point, as the level of intake is increased, the percentage stored is increased.

The evidence in the literature regarding sex differences in ability to store vitamin A is conflicting. Lemley et al. ('47) reported that there is no sex difference. The results of our study support the observation of Brenner et al. ('42) that under comparable experimental conditions females store appreciably more vitamin A in the liver than males. The addition of lecithin to the ration enables either sex to store greater amounts of vitamin A in the liver when either 5 μ g of vitamin A ester or 20 μ g of carotene are fed daily.

SUMMARY

A series of 3 experiments were undertaken to study the effects of crude soya lecithin and choline on the absorption, utilization and storage of vitamin A in laboratory rats. The following pertinent observations were made.

When 2% of the basal vitamin A-free diet consisted of lecithin ($\frac{1}{2}$ or $\frac{1}{4}$ of the fat replaced by lecithin), greater weight gains during the depletion period were obtained than when 0.07% choline or a similar diet without added lecithin was fed. Limited data indicate that liver reserves of vitamin A were exhausted slightly more rapidly when the depletion diet contained added lecithin.

The feeding of lecithin during the depletion period had a slight, although not a statistically significant, carry-over effect in increasing the weight gains during the vitamin A repletion period. On the other hand, choline feeding during depletion resulted in significantly smaller weight gains during the following vitamin A repletion period.

The feeding of lecithin with a vitamin A or carotene supplement resulted in greater gains in weight and increased liver storage of vitamin A. When choline was fed with these supplements, the liver storage of vitamin A was no different from that of the groups which received the vitamin supplement alone.

At the levels fed, there was a greater percentage storage of vitamin A when β -carotene was the supplement, although the weight gains of the vitamin A-supplemented groups were greater.

Females consistently stored more of the vitamin in the liver under all the dietary conditions studied.

The data indicate that lecithin enhances both the absorption and utilization of vitamin A and carotene. The choline fraction of the lecithin molecule is not responsible for this effect.

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DISTRIBUTION AND FRACTIONATION OF THE MONKEY ANTI-ANEMIA FACTOR¹

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ONE FIGURE

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Extensive studies in our laboratory on the nutritional requirements of the rhesus monkey (*Macaca mulatta*) have shown that this species will grow at a normal rate for extended periods of time when given a purified ration supplemented with all of the known crystalline B vitamins. However, when individual vitamin deficiencies were studied, it was found that the monkey frequently would fail to respond completely to the administration of the missing vitamin. This was particularly evident in the case of riboflavin, pyridoxine, pantothenic acid and folic acid deficiencies (Cooperman et al., '45; McCall et al., '46; Cooperman et al., '46a). The inclusion of unenriched corn grits in the basal ration at a level of 40% also precipitated the condition (Cooperman et al., '46b). The anemia as well as the lack of growth resulting from these deficiencies was counteracted by certain crude materials such as liver and milk. Consequently, it was postulated that a new factor was involved, and it was tentatively named the monkey anti-anemia factor (Cooperman et al., '45). The assay procedure developed for measuring this factor consisted of omit-

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ting a vitamin such as riboflavin from the regimen until the monkey had lost weight and developed an anemia. At this time, vitamin therapy was instituted with the result that the monkey would respond suboptimally to the vitamin. If a preparation fed at this point would bring about a weight and hematological response, it was concluded that it contained the new factor. In the case of the corn grit ration, the condition was precipitated even in the presence of all B vitamins. Administering active preparations brought about a weight and hematological response comparable to that observed on the vitamin deficient rations.

We are reporting in this paper studies on potential sources of the anti-anemia factor, fractionation procedures and possible methods for improving the assay procedure.

EXPERIMENTAL

The housing, care and handling of the monkeys have been described previously (Waisman et al., '43). The basal ration had a percentage composition of sucrose 73, purified casein 18, salts IV 4, corn oil 2 and cod liver oil 3, with 40% of the "dry mix" replaced by corn grits (Cooperman et al., '46b). Adequate amounts of thiamine (1 mg), riboflavin (1 mg), pyridoxine hydrochloride (1 mg), niacin (5 mg), pantothenic acid (3 mg), choline chloride (25 mg), i-inositol (50 mg), p-aminobenzoic acid (50 mg), biotin (0.02 mg), folic acid (0.1 mg), and ascorbic acid (50 mg) were fed daily as a separate supplement.

Monkeys placed on the corn grit ration grew normally for 3-10 months before showing deficiency symptoms. Eventually a plateau or loss in weight occurred, an anemia developed and the animals died unless further supplementation was given. In the assay procedure, it was required that all animals show a plateau or weight loss for at least 1 month before administration of the test material. A 1-month interval was generally allowed in order to ascertain the activity of the sample as evidenced by a weight response and increase in hemoglobin concentration of the blood. Various substances were assayed using this technique, and it was found that fresh liver or whole

dry liver substance as well as malt sprouts and milk constituted the best sources of the factor (Ruegamer et al., '47a). Furthermore, upon preliminary fractionation of milk, it was found that the activity occurred in the whey fraction (Cooperman et al., '46c).

On the basis of this work, a sample of whey solids² was fed to 1 monkey at a level of 3% of the basal ration. The initial weight of the animal was 5020 gm and the hemoglobin 12.6 gm %. Since no response was seen after the first week, the level of whey solids was increased to 6%. No response was observed at the end of the third week, and the preparation was discontinued (table 1). An active liver preparation was then fed for 1 month and the weight of the animal increased to 5400 gm, and the hemoglobin rose to 13.7 gm %.

Since our regular monkeys were frequently kept on a ration consisting mainly of raw potatoes and carrots and seemed to remain in a fair state of health, 50 gm of raw potatoes and carrots were fed to 2 deficient monkeys, respectively. The raw potato failed to produce a response, whereas the addition of a liver preparation did produce a gain of 540 gm in 2 months. The carrot supplementation likewise failed to produce any increase in either body weight or hemoglobin (table 1).

In the microbiological assay with *Streptococcus faecalis* (American type culture collection no. 8043), whole yellow corn was found to give a greater growth stimulation than corn grits (Ruegamer et al., '47a; Cooperman et al., '46d). The activity measured by the *S. faecalis* assay was originally thought to parallel that of the monkey anti-anemia factor. Consequently 20% whole yellow corn was fed to 1 monkey at the expense of the corn grits in the basal ration. No activity could be seen as evidenced by the failure to produce either a growth or hemoglobin response in the monkey (table 1). Likewise, yeast extract³ which was active for the microorganism, was fed to 1 monkey at levels as high as 6% without

² Western Condensing Company, Appleton, Wis.

³ Difco.

TABLE 1

Data showing growth and hemoglobin responses to various preparations tested for the monkey anti-anemia factor.

EXPT. NO.	MONKEY NO.	PREPARATION	DURATION OF TEST	INITIAL WT.	FINAL WT.	INITIAL Hb.	FINAL Hb.
				gm	gm	gm %	gm %
1	249	6% whey solids	3 wks.	5020	5100	12.64	12.33
	340	Acetone extr. \approx 5 gm W.L.S. ¹	1 mo.	3580	3920	12.91	14.0
2	295	50 gm raw potato/day	3 wks.	4600	4650	11.63	10.43
	295	Norite eluate \approx 18 gm fresh liver/day	3 mo.	4700	5240	8.82	12.76
3	297	50 gm raw carrot/day	3 wks.	4640	4680	10.6	10.01
4	189	20% whole yellow corn	1 mo.	5400	5460	13.3	13.38
5	296	6% Difco yeast extr.	1 mo.	3880	3940	12.33	12.14
	296	Alc. extr. liver \approx 9 gm/day	1 mo.	3930	4380	11.93	13.89
6	341	0.5% L-lysine plus 0.5% DL-tryptophane	1 mo.	3850	3900	12.91	13.17
7	277	0.5 mg folic acid/day	3 wks.	4220	4230	13.38	12.06
8	248	50 mg rutin + 50 mg hesperitin per day	1 mo.	5060	5120	..	.
9	276	1 U.S.P. unit/day of reticulogen injected	3 wks.	3260	3300	13.38	13.23
	277	15 units/day injected	3 wks.	3300	3300	13.03	13.03
	276	45 units/day injected + 0.25 mg B ₆ conjugate	2 wks.	3500	3570	13.30	13.03
	277	45 units/day orally	3 wks.	3280	3280	12.56	10.89
	276	15 units/day + 24% casein	1 mo.	4200	4200	12.06	12.14
	277	15 units/day orally + B ₆ conj. (0.25 mg)	2 wks.	3300	3280	13.03	12.56
10	276	1 gm fresh liver/day	2 wks.	5000	4950	14.28	14.28
	276	3 gm fresh liver/day	3 wks.	4950	5050	14.28	14.55
	276	4 gm fresh liver/day	2 wks.	5050	5200	14.55	14.55
	297	4 gm fresh liver/day	3 wks.	3830	4300	10.43	13.03
11	249	60% alc. extra. \approx 4 gm liver/day	3 wks.	4960	5000	13.03	12.64
	368	Alc. extr. \approx 6 gm liver/day	2 wks.	3240	3300	12.76	12.76
	368	\approx 9 gm liver/day	1 mo.	3300	3680	12.76	13.88
	277	\approx 9 gm liver/day	5 wks.	3280	3820	10.89	14.08
	296	\approx 9 gm liver/day	1 mo.	3930	4380	11.93	13.89
	340	\approx 9 gm liver/day	3 wks.	4680	5000	13.38	14.97
12	276	60% Alc. extr. malt sprouts \approx 10 gm/day	1 wk.	3620	3620	13.30	...
	276	\approx 30 gm/day	2 wk.	3620	3650	13.30	13.03
	276	\approx 50 gm/day	1 mo.	3650	3820	13.03	13.4
13	277	Alc. extr. tomatoes \approx 20 gm/day	2 wks.	4220	4250	14.08	...
	277	\approx 40 gm/day	1 wk.	4250	4200	14.08	13.38
	297	\approx 60 gm/day	2 wks.	4700	4750	12.06	10.09
14	340	Acetone extr. \approx 5 gm W.L.S./day	1 mo.	3580	3920	12.5	16.3
15	297	Norite eluate \approx 12 gm liver/day	3 wks.	4430	4430	12.56	10.66
	297	\approx 18 gm/day	3 wks.	4430	4640	10.66	11.21
	295	\approx 18 gm/day	1 mo.	4900	5200	8.82	12.76

¹ Whole liver extract.

any beneficial effect. An active liver preparation fed after the yeast was removed from the ration brought about a weight response of 450 gm and a hemoglobin response from 11.93 gm to 13.89 gm % in 1 month (table 1 and fig. 1, monkey 296).

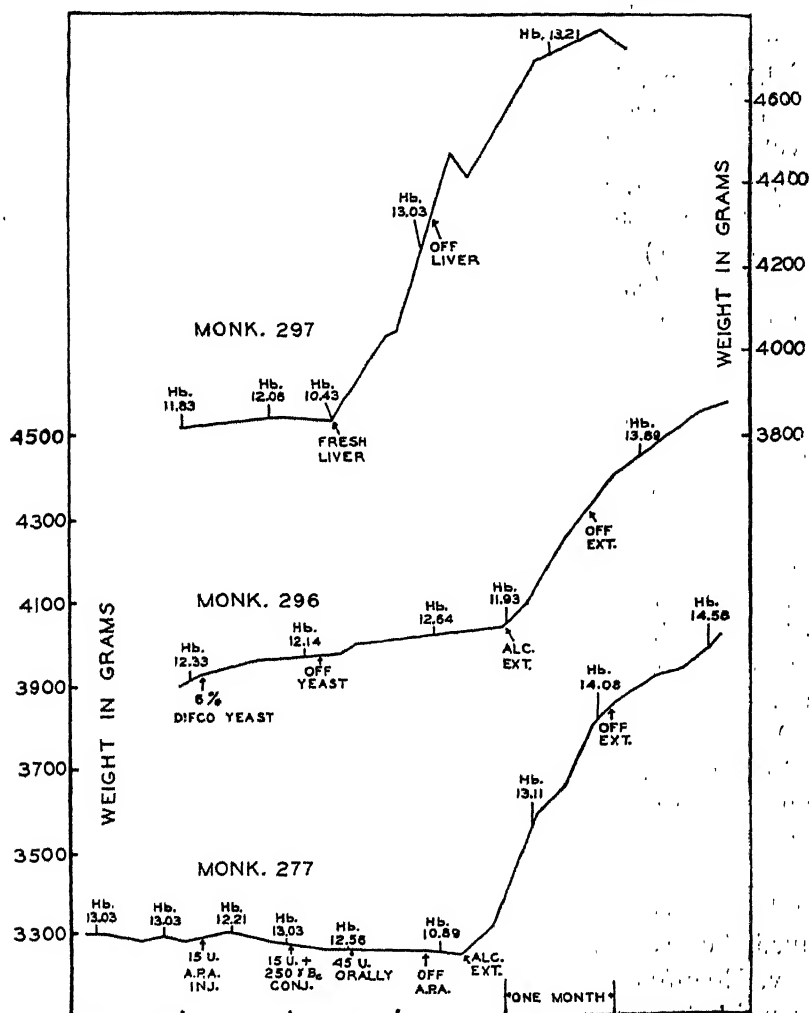


Fig. 1 Typical growth curves and hemoglobin values for 3 monkeys receiving liver preparations. The alcohol extract indicated on the curves was prepared from fresh liver and fed by stomach tube at a level ≈ 9 gm per day. The fresh liver homogenate given monkey 297 was fed at a level of 4 gm per day.

Since the inclusion of 40% corn grits lowered the lysine and tryptophane contents of the casein ration, 0.5% L-lysine and 0.5% DL-tryptophane were fed as supplements mixed in the basal ration. However, no response could be seen in a deficient monkey and this supplementation was discontinued (table 1).

In order to determine if the folic acid level (0.1 mg/day) might be low, daily supplements of 0.5 mg of folic acid were fed to 1 monkey. No effect could be seen and the original level of 0.1 mg/day was continued (table 1).

When 50 mg of rutin and 50 mg of hesperidin were fed daily to a deficient monkey for 1 month, no response could be seen (table 1).

Concentrates of the pernicious anemia factor have been found active for the dog, rat, chicken and microorganism *S. faecalis* (Ruegamer et al., '47b; Sporn et al., '47; Bethel et al., '47; Nichol et al., '47; unpublished data). Samples of reticulogen⁴ were injected intramuscularly into 2 monkeys at levels ranging from 1 to 45 U.S.P. units per day. No apparent effect could be seen either in hemoglobin or in weight. The concentrates were then fed orally at the same levels, but without effect. Thinking that the factor might in some way be a protein utilization factor, the extracts were given with a 24% casein basal ration. B₆ conjugate was also given simultaneously with the pernicious anemia preparation, but neither combination showed any activity (table 1 and fig. 1, monkey 277).

On the basis of these results, it was decided to fractionate some of the more active materials. In all cases the preparations were fed by stomach tube because of the unpalatability of some of them for the monkey. First, the approximate minimal level of fresh liver which would give a response was determined. Accordingly, 1 gm of fresh liver was given to 1 monkey daily. No effect could be seen after 2 weeks so the level was increased to 3 gm per day. A slight response was seen, but when the level was raised to 4 gm, a good response of 150 gm gain in body weight followed. A liver homogenate was given to an-

⁴ Eli Lilly and Company, Indianapolis, Ind.

other monkey at a level of 4 gm per day, and this animal showed a response of 470 gm in weight and 2 gm % in hemoglobin in 3 weeks (fig. 1, monkey 297). Therefore, it was concluded that between 3 and 4 gm of fresh liver is the minimal amount necessary for a good response.

An ethanol extract was made from fresh beef liver by grinding the liver in a meat grinder and adding 95% ethanol until the ethanol concentration was 60%. The suspension was stirred for 1 hour and allowed to stand overnight. The extract was then removed and the residue re-extracted with 60% ethanol. The extracts were combined and filtered through filter cell. After removing the ethanol by vacuum distillation, the preparation was extracted with ether and the ether layer was discarded. The aqueous phase was concentrated further to a 10:1 concentration and stored at 0°C. This extract was fed by stomach tube at a level equivalent to 4 gm of fresh liver per day. No effect could be seen in 2 weeks so the level was raised to the equivalent of 6 gm of fresh liver. Still no effect was observed and the level was further increased to the equivalent of 9 gm of fresh liver per day. At this level, a weight response of 380 gm and a hemoglobin response of 1 gm % was seen in 1 month. Three other monkeys were given the extract at a level equivalent to 9 gm of fresh liver and similar responses were obtained (table 1). Therefore it was concluded that the ethanol extract was active at a level representing 9 gm of fresh liver per day. Furthermore, dry weight analysis showed that when 4 gm of fresh liver homogenate were fed, between 0.8 and 1 gm of solids was furnished per day. When the alcohol extract was fed at a level equivalent to 9 gm of fresh liver, only 270–300 mg of solids were furnished per day.

Methanol extracts of fresh beef liver prepared in the same manner were also active. However, when ethanol extracts were prepared from malt sprouts and fresh tomatoes, only borderline activity could be observed even when levels up to that equivalent to 50 gm of the original material were fed per

day. Aqueous extracts had been found to be quite active at lower levels (table 1).

An acetone extract was prepared by extracting whole liver substance⁵ in a Soxhlet extractor for 24 hours. When this preparation was fed at a level the equivalent of 5 gm per day, the weight of the test animal increased 440 gm in 1 month and the hemoglobin increased from 12.91 to 14 gm % as seen in table 1.

On the basis of studies carried out with the rat (Jaffé and Elvehjem, '47), a norite eluate fraction from fresh liver was prepared and fed as a possible source of the monkey factor. The preparation was made from the methanol extract previously described by making the extract acid with concentrated HCl to methyl orange (red) and filtering. Norite⁶ was added (20 gm/kg liver) and the solution was stirred for 1 hour. The solution was filtered in a Buchner funnel using a pad of filter cell. The adsorption procedure was repeated and the norite was washed with 50% methanol while still on the Buchner, the filtrate being discarded. Without suction, 150 ml of 1% phenol solution were added to the Buchner containing the norite, and the solution was allowed to drain for 1/2 hour. After stirring carefully, suction was applied and the filtrate collected. Approximately 8 elutions were carried out in this manner and the eluates were combined and extracted with ether until free from phenol. The eluate was then distilled under vacuum until a concentration equivalent to 60 gm of fresh liver per milliliter was obtained. When the eluate was fed to 2 monkeys at levels as high as that equivalent to 12 gm of fresh liver per day, no activity could be observed. If the level was increased to one equivalent to 18 gm of fresh liver, good responses in weight and hemoglobin were obtained in both monkeys (table 1). Dry weight analysis revealed that approximately 90 mg of solids were being fed per day as compared to 1 gm solids when fresh liver was used and 270-300 mg solids when the ethanol extract was used.

⁵ Wilson and Company, Chicago, Ill.

⁶ Dareo G-60.

Since between 3 and 10 months are generally required to precipitate the deficiency in the monkey, several attempts were made to develop a more rapid means of estimating the activity of various preparations. A microbiological method using *S. faecalis* was tried in the hope that the activity observed with *S. faecalis* would parallel that seen with the monkey. As the study progressed, however, it was found that the microörganism and the monkey were not responding to the same factor. Therefore the microbiological assay was discontinued and alterations in the diet of the monkey were tried. Since better results had been obtained in the rat assay on a natural ration as compared to a purified diet (Sporn et al., '47), a 50-50 corn-soybean ration with added salts (2%), vitamins and cystine was fed to 2 monkeys. These animals found the ration unpalatable; therefore, the experiment was discontinued.

Because of early successes in showing deficiencies in rats by feeding sulfa drug, 1% sulfasuxidine was included in the ration of 1 monkey for a period of 2½ months. During this time the monkey gained 350 gm and showed no depression in the hemoglobin level.

The protein level was reduced in the case of another monkey to 12% casein. This animal continued to grow approximately 300 gm over a 3-month period so the regimen was discontinued.

Because of results obtained in dog studies involving the relationship of folic acid to fat metabolism, 4 monkeys were fed a fat free ration in which the fat of the basal purified diet was replaced by sucrose. Two of these monkeys, furthermore, received no folic acid whereas the other 2 received adequate amounts of folic acid. After 2 months on this regimen, the monkeys were still growing slowly, and no significant blood changes occurred.

None of the proposed procedures for shortening the assay has been found to be successful, but this phase of the work is being continued.

DISCUSSION

When a sample of commercial whey solids was fed at a level of 6%, no activity could be obtained for the monkey anti-anemia factor. Apparently the factor was destroyed in the processing of the whey since fresh whey was found to possess full activity.

Fresh samples of raw carrots, raw potatoes, corn and soybeans were fed as sources of the monkey factor, but all were found to be inactive even when fed at levels 10 times that of liver. Liver still remained the best starting material for fractionation work.

High levels of folic acid and pernicious anemia preparations also failed to show activity. From these data it may be concluded that the monkey factor is separate from the rat, chicken and dog factors since all 3 of these species responded to pernicious anemia concentrates.

When liver extracts were employed, it became necessary to give the preparations by stomach tube as the monkeys found them unpalatable. This technique of administering the liver preparations in single doses may be open to criticism in view of newer theories which propose that for optimum growth, all essential nutrients must be present within the animal at the same time. However, since good responses were observed using this procedure, we believe it to be a satisfactory method for giving the preparations.

Approximately 4 gm of fresh liver are needed per day when given by stomach tube. When a 60% methanol or ethanol extract of fresh liver was prepared and fed to monkeys, good responses could be obtained when equivalents of 8 or 9 gm of fresh liver were fed per day. This would mean that approximately $\frac{1}{2}$ of the activity was lost by this step, but the solids decreased from 800–1000 mg found in 4 gm of fresh liver to 270–300 mg contained in the alcohol extract when fed at a level equivalent to 9 gm per day. Thus a threefold concentration was obtained by extracting the fresh liver with alcohol. In the case of the norite eluate, the equivalent of 18 gm of fresh liver was needed. However, dry weight analysis re-

vealed that the solids furnished by this equivalent amount of fresh liver amounted to only 90 mg per day. Therefore, an overall approximate tenfold concentration was realized by the norite elution procedure.

SUMMARY

Further studies were carried out on the distribution and fractionation of the monkey anti-anemia factor. Whey solids, yellow corn, raw potato and raw carrot are poor sources of the factor. Higher levels of folic acid and rather high levels of rutin and hesperidin, L-lysine and DL-tryptophane, and pernicious anemia concentrates failed to show any activity for the monkey.

Approximately 4 gm of fresh liver are needed by the monkey per day. An ethanol extract of fresh liver was active as well as a methanol and acetone extract of liver. A norite eluate prepared from the alcohol extract also proved active when fed at a higher level. A tenfold concentration was observed by the norite elution method.

Attempts were made to shorten the assay by feeding sulfasuxidine in the ration, lowering the protein intake, and by feeding fat free rations.

ACKNOWLEDGMENTS

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FURTHER STUDIES ON THE RELATIONSHIP OF NICOTINIC ACID, TRYPTOPHANE AND PRO- TEIN IN THE NUTRITION OF THE PIG¹

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The requirement of the pig for nicotinic acid has been observed by many workers; however, Wintrobe and co-workers ('45) reported that nicotinic acid deficiency could not be produced in young pigs fed a purified ration containing 26% casein. In a paper previous to the present one Luecke et al. ('47) reported that nicotinic acid deficiency can be produced in young pigs fed a low protein ration composed largely of corn, and that supplements of tryptophane were effective in preventing this deficiency. It was noted, however, that daily supplements of 200 mg of DL-tryptophane per pig were not completely effective in preventing the characteristic intestinal lesions of nicotinic acid deficiency. With this in mind, it seemed worthwhile to determine whether higher levels of tryptophane would be effective in completely preventing nicotinic acid deficiency. Furthermore, since Powick and co-workers ('47) were not able to confirm the work of Wintrobe et al. ('45) regarding the production of nicotinic acid deficiency using rations containing high levels of casein, it was

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decided to determine whether the deficiency symptoms could be produced in a high protein ration made up largely of corn.

EXPERIMENTAL

Pigs used in this experiment were farrowed by Duroc Jersey sows which had been bred to a Yorkshire boar. Two weeks before the pigs were placed on the experiment they were vaccinated for hog cholera, using the serum and virus method. The 3 basal rations used are shown in table 1. The crude protein content of rations A, B, and C was 15.1, 19.4 and 29.6% respectively, and the nicotinic acid content was 11.2, 8.7 and

TABLE 1
Composition of rations¹

	RATION		
	A	B	C
	%	%	%
Corn	87	80	68
Casein (commercial)	5.5	12	25
Soybean oil meal	5.5	6	5
Complex mineral mixture	2	2	2

¹ The following amounts of vitamins were supplied daily to each animal: thiamine, 10 mg; riboflavin, 10 mg; calcium pantothenate, 25 mg; and pyridoxine, 6 mg.

8.1 mg per pound respectively, as determined microbiologically by the method of Krehl, Strong and Elvehjem ('43). It can be seen that the nicotinic acid content of the 3 rations decreased as the level of casein increased, due to the fact that commercial casein is extremely low in nicotinic acid. This fact serves a very useful purpose since, even though the higher levels of casein stimulate greater feed consumption, the actual intakes of nicotinic acid are quite uniform.

The tryptophane content of rations A, B, and C was 0.13, 0.20 and 0.41% respectively, as determined microbiologically using the organism *Lactobacillus arabinosus*. The medium used for the tryptophane assay was essentially the same as

that described by Sauberlich and Baumann ('46), and the samples were prepared for assay according to the method of Wooley and Sebrell ('45). The 3 rations were supplemented with vitamins A and D in amounts which supplied 2,000 I.U. of vitamin A and 200 I.U. of vitamin D per pound of feed. Thiamine, riboflavin, calcium pantothenate and pyridoxine were fed daily to each pig in the amounts indicated (table 1). The composition of the complex mineral mixture was the same as that described previously (Luecke et al., '47).

At the conclusion of the 6-week experimental period the animals were autopsied and pathological studies made of the affected tissues.

RESULTS

Pigs in lot 1 were fed the low protein ration A plus daily supplements of 30 mg of nicotinic acid. The growth response of these pigs is shown in table 2. The animals in this lot

TABLE 2
*Response of pigs fed the various experimental rations
(Five pigs in each lot on trials lasting 6 weeks)*

	LOT				
	1	2	3	4	5
Ration fed	A ¹	A	A ²	B	C
Initial age in weeks	5	5	5	6	5
Average initial weight (lbs.)	22	22	22	27	22
Average daily gain (lbs.)	0.61	0.26	0.84	0.74	1.05
Average daily feed consumption (lbs.)	1.37	1.05	1.75	2.00	2.06
Lbs. of feed per lb. of gain	2.25	4.04	2.08	2.70	1.96

¹ Pigs in lot 1 received daily supplements of 30 mg of nicotinic acid.

² Pigs in lot 3 received daily supplements of 1 gm of DL-tryptophane.

gained an average of 0.61 pounds per day while consuming 1.37 pounds of feed. The growth response of these pigs was rather surprising, inasmuch as the protein content of ration A was lower than is generally recommended for pigs of that age and weight. The pigs in this lot required 2.25 pounds of feed to produce 1 pound gain in body weight.

Pigs in lot 2 were fed ration A. During the 4th week typical symptoms of nicotinic acid deficiency were noted. These included diarrhea, a rough haircoat and lowered appetite. The intestinal lesions of nicotinic acid deficiency have been previously described (Luecke et al., '47). The pigs in this lot required 4.04 pounds of feed to produce 1 pound of gain.

Ration A plus daily supplements of 1 gm of DL-tryptophane were fed to the pigs in lot 3. In a previous experiment (Luecke et al., '47) daily supplements of 200 mg of DL-tryptophane produced good growth, but did not entirely prevent the typical intestinal lesions of nicotinic acid deficiency. Inasmuch as it seemed probable that the 200 mg level was too low, the supplementary level of tryptophane was increased to 1 gm. The growth rate of the pigs in this lot was excellent (table 2). These pigs gained an average of 0.84 pounds per day while consuming 1.75 pounds of feed, and it required only 2.08 pounds of feed to produce 1 pound of gain. No intestinal lesions characteristic of nicotinic acid deficiency were found in the animals of this lot upon subsequent autopsy. This indicates that adequate levels of tryptophane will completely prevent symptoms of nicotinic acid deficiency in the pig.

Pigs in lot 4 were fed ration B and gained an average of 0.74 pounds per day (table 2), which is somewhat better than the daily gains produced by the animals in lot 1. However, the pigs in lot 4 required 2.70 pounds of feed to produce 1 pound gain in body weight as against 2.25 pounds for the animals in lot 1. During the 5th week of the experiment 2 of the pigs in lot 4 developed diarrhea, and upon autopsy at the conclusion of the experiment 3 animals showed evidence of enteritis; the remaining 2 pigs were normal. The intestinal lesions found at autopsy in the 3 pigs of this lot were not as severe as those found in the animals of lot 2 fed low protein ration A. However, it seemed evident that even though the crude protein content of ration B was high, it did not contain sufficient tryptophane to counteract completely the low nicotinic acid content.

Pigs in lot 5 were fed ration C. The growth response of these animals was excellent (table 2). They gained weight more rapidly and efficiently than any other experimental lot, requiring only 1.96 pounds of feed to produce 1 pound gain in body weight. No evidence of nicotinic acid deficiency was noted, either during the experiment or upon subsequent autopsy.

Luecke and coworkers ('47) have shown that one of the symptoms of nicotinic acid deficiency in the pig is a low urinary excretion of N¹-methylnicotinamide. Accordingly, during the 6th week of the experiment the pigs were placed

TABLE 3

Average daily intakes of tryptophane and nicotinic acid and excretion of N¹-methylnicotinamide

LOT	TRYPTOPHANE		NICOTINIC ACID		N ¹ -METHYL-NICOTINAMIDE
	In feed	Supplement	In feed	Supplement	
	<i>gm</i>	<i>gm</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>
1	0.80		15.34	30	13.87
2	0.61		11.76		4.62
3	1.12	0.50 ¹	19.60		18.14
4	1.80		17.40		9.83
5	3.80		16.70		15.06

¹ Assuming D-tryptophane to be completely inactive.

in metabolism cages and a 24-hour collection of urine obtained. The average daily excretions of N¹-methylnicotinamide, as determined by the method of Huff, Perlzweig and Tilden ('45) are shown in table 3. It should be noted that the average excretion of N¹-methylnicotinamide for the pigs in lot 2 was considerably lower than that of lot 4. This agreed with the results obtained at autopsy in that the intestinal lesions found in pigs of lot 2 were far more severe than those found in lot 4.

Since the nicotinic acid and tryptophane contents of rations A, B and C were known, the average daily intakes of these substances could be calculated from the average daily

feed consumption. The results of these calculations are shown in table 3. It seems evident that the addition of nicotinic acid to the low protein ration A increased the utilization of dietary tryptophane, since the animals in lot 1 grew normally on an average daily intake of 0.80 gm of tryptophane, while in the case of lot 4 the daily ingestion of 1.80 gm gave only a slightly better growth rate and, as a matter of fact, resulted in a lower efficiency of feed utilization. The fact that the supplementation of ration A with DL-tryptophane resulted in good growth indicates that tryptophane is the limiting amino acid in this ration. On the basis of tryptophane intake it is rather surprising that the pigs in lot 3 grew more rapidly and efficiently than the animals in lot 4. If it is assumed that D-tryptophane is inactive for the pig, then the animals in lot 3 were actually consuming less of this amino acid than the pigs in lot 4. Although the present experiment offers no proof, it seems possible that the pig may be able to utilize D-tryptophane to some extent. Another possibility is that the addition of synthetic tryptophane stimulates nicotinic acid synthesis which, in turn, improves tryptophane utilization. This possibility has already been suggested by Krehl et al. ('46).

DISCUSSION

In the experimental production of nicotinic acid deficiency in pigs, the age and weight of the pig are of great importance. It has been the authors' experience that symptoms of nicotinic acid deficiency are produced more readily in pigs weighing less than 30 pounds. Furthermore, we have never been able to produce nicotinic acid deficiency in pigs weighing 60 pounds or more when fed a low protein ration made up largely of corn. Braude, Kon and White ('46) were also unable to produce nicotinic acid deficiency in older pigs.

The fact that nicotinic deficiency could not be produced on a corn ration containing 25% casein confirms the work of Wintrobe et al. ('45). Although the pigs receiving the high protein ration were ingesting 16.7 mg of nicotinic acid per day (table 3), it is not likely that this amount of nicotinic acid

would prevent deficiency, since in the case of lot 4 the pigs ingested 17.4 mg per day and showed mild deficiency symptoms.

Since the nicotinic acid requirement of the pig is directly related to the tryptophane intake, the difficulties encountered in attempting to elucidate the requirement for this vitamin are obvious. The use of purified rations in estimating the nicotinic acid requirement of the pig (Hughes, '43) will probably give results lower than those obtained by using a ration made up largely of corn.

SUMMARY

Adequate amounts of DL-tryptophane as supplements to a low protein corn ration will prevent nicotinic acid deficiency in the pig. Supplementation of the same low protein ration with nicotinic acid seems to increase the utilization of dietary tryptophane.

No symptoms of nicotinic acid deficiency were produced on a corn ration containing 25% casein. Moreover, pigs fed this high protein ration grew very rapidly and required only 1.96 pounds of feed to produce 1 pound gain in body weight.

Mild symptoms of nicotinic acid deficiency were produced in pigs whose calculated average daily intake of nicotinic acid was 17.4 mg.

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RESPONSE OF DOGS TO LIVER EXTRACTS CONTAINING THE PERNICIOUS ANEMIA FACTOR¹

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FOUR FIGURES

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Niacin was first shown to be essential for the dog through the use of the modified Goldberger diet (Elvehjem et al., '38). Schaefer et al. ('42) found that young puppies developed symptoms of blacktongue in 14–18 days when niacin was omitted from a purified ration containing sucrose, casein, cottonseed oil and cod liver oil supplemented with thiamine, riboflavin, pyridoxine, pantothenic acid and choline. The syndrome could be counteracted by the administration of niacin, but incomplete responses were observed in certain cases.

West ('41) first observed that sulfapyridine inhibited the curative effect of niacin in deficient dogs and that fresh liver counteracted this inhibition. Schaefer et al. ('42) repeated this work using the purified ration described above and found that the sulfapyridine inhibition could not be overcome by nicotinamide, dried liver or liver extract powder.

Krehl et al. ('45, '46) found that a more consistent response to niacin could be obtained when folic acid was added to the purified rations. Furthermore, these workers found that fresh

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liver and milk were effective in counteracting the sulfapyridine-induced inhibition in niacin-deficient animals.

Rhoades and Miller ('33) studied the anemia associated with blacktongue and found that a progressive macrocytic anemia developed in dogs which were failing on deficient diets. Handler and Featherston ('43) demonstrated the same type of anemia in dogs by feeding a modified Goldberger diet, the corn meal diet of Koehn and Elvehjem ('37) and a purified diet. The anemia in the case of dogs on the 1st two diets responded well to the administration of niacin or nicotinamide. However, the animals receiving the purified diet showed only suboptimal responses to niacin (10-12 gm % hemoglobin). Consequently, these workers suggested that other factors might be involved.

Krehl et al. ('45, '46) found that even though folic acid helped to produce more consistent responses to niacin on a niacin-deficient ration, the anemia which prevailed was little affected by the presence of folic acid. Because complete responses were not obtained with folic acid, these workers also suggest the possibility that another factor is involved.

In this report, we wish to present further evidence that an additional factor is required by the dog when maintained on a niacin-deficient diet. We also wish to present data to show that commercial pernicious anemia preparations are very rich sources of the factor.

METHODS

Approximately 35 weanling mongrel dogs were used in these experiments. They were dewormed and freed of external parasites at the beginning of the experiment, and were placed in individual cages equipped with heavy wire mesh bottoms. All animals were given the basal ration and water ad libitum. The basal ration consisted of sucrose 65%, alcohol-extracted casein 19%, cottonseed oil 11%, salts IV 4% and sulfasuxidine 1%. Some modifications in the composition of the basal ration were made, but these are indicated in each case. The following crystalline B vitamins were fed as sup-

plements to the basal ration: 0.1 mg each of thiamine and riboflavin, and 0.6, 0.5 and 25 mg, respectively, of pyridoxine hydrochloride, calcium pantothenate and choline chloride, per kg of body weight per day. The vitamins were prepared as an aqueous suspension at such a concentration that the suspension could be fed at the rate of 1 ml per kg of body weight per day on every 3rd day. In addition, 1800 I.U. of vitamin A and 500 I.U. of vitamin D per day were given.²

Blood samples (3 ml) were taken routinely from the radial vein, and hemoglobin values, red cell counts and hematocrit values were determined. From these data, the mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration were calculated.

EXPERIMENTAL AND RESULTS

Young growing dogs lost weight when placed on a niacin-deficient ration, and niacin therapy became necessary in 14-18 days. In most cases, the animals gained between 800 and 1000 gm when given a single dose of 25 mg of niacin. This weight response was only temporary, however, and the animals soon began to lose weight again. The administration of a 2nd dose of 25 mg of niacin brought about weight responses in a number of dogs, but eventually a point was reached where all animals failed to respond to niacin (fig. 1). Unless further therapy was given, death resulted in these animals. The number of responses to single doses of niacin varied between 2 and 5 per dog. When the animals failed to respond to niacin, blood data revealed that an anemia persisted (8-12 gm % hemoglobin, 2-3 million red cells and hematocrit values of 20-30%). These data, which were collected from 20 animals, were used to calculate the mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration. Similar blood data from 20 control animals receiving niacin from the beginning of the experiment were taken simultaneously and the same calculations were made. These are summarized in table 1.

² Vitamin A and vitamin D given in the form of Haliver oil and Drisdol.

From these data it is clear that a macrocytic anemia existed in the animals which failed to respond to niacin.

Since folic acid had been shown to play a part in the response of dogs to niacin, it was fed at a level of 0.1 mg per day to 6 animals which had failed to respond to niacin. Some of the animals showed weight responses as a result of the folic

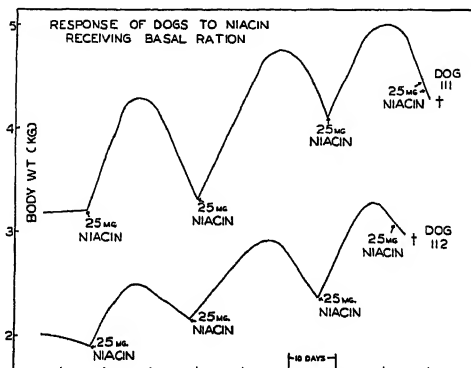


Fig. 1 Failure of dogs to respond to niacin.

TABLE 1

Comparison of blood pictures of control animals and depleted animals failing to respond to niacin

	MEAN CORP. VOLUME	MEAN CORP. HEMOGLOBIN	MEAN CORP. HEMOGLOBIN CONCENTRATION
	mm ³	μg	%
Control animals	69	22.4	32.1
Depleted animals	82	27.5	33.8

acid therapy, but little if any change could be observed in the blood picture (table 2). Several animals developed a flaccid type of paralysis and died. An animal afflicted with typical paralysis is shown in figure 2. Whether or not this paralysis is related to that seen in humans suffering from pernicious anemia has not been determined. In general, the longer the animal can be maintained in the deficient state, the more severe the symptoms become. It is exceedingly difficult to

prolong the deficiency, however, since most of the dogs in this condition die very suddenly.

Since the paralysis and the anemia were similar to the symptoms encountered in pernicious anemia, and since stubborn cases of pellagra in the human are sometimes treated successfully with liver extracts, commercial preparations of the pernicious anemia factor were tried. Several dogs which

TABLE 2

Effects of folic acid and antipernicious anemia concentrates (A.P.A.) upon dogs failing to respond to niacin

DOG NO.	FOLIC ACID THERAPY					A. P. A. THERAPY		
	Body weight		Duration of test	Hemoglobin		Weight after therapy	Duration of test	Hemoglobin after therapy
	Before therapy	After therapy		Before therapy	After therapy			
	kg	kg	days	gm%	gm%	kg	days	gm%
114 ¹	9.2	9.0	30	10.89	11.1	10.5	60	15.15
120 ¹	9.1	6.5	18	11.71	11.28	+		
121 ¹	6.9	10.0	30	10.89	10.35	—		
419 ¹	8.6	8.4	5	11.55	11.55	+		
445 ¹	7.2	9.5	30	9.13	9.73	11.6	30	13.7
451 ¹	6.3	7.3	35	12.21	12.76	8.6	30	15.29
	A. P. A. THERAPY					FOLIC ACID THERAPY		
119 ²	7.25	10.0	37	10.28	11.21	10.5	30	14.6
118 ³	6.8	7.8	60	12.9	13.0	8.9	28	15.8
204 ²	8.7	16.2	60	10.45	12.0	20.2	59	15.0

¹ Dogs 114, 120, 121, 419, 445, and 451 each received 1 U.S.P. unit of reticulogen (Lilly) injected per day.

² Dogs 119 and 204 received 10 and 7 U.S.P. units respectively of reticulogen per day.

³ Dog 118 received 7 U.S.P. units of Sharp and Dohme no. 2505 per day, and was given B₁₂ conjugate instead of free folic acid.

had failed to respond to niacin on a niacin-deficient ration were given adequate amounts of niacin and folic acid. Little weight and no blood responses were seen. These animals were required to show a weight and hematological plateau for a considerable length of time (approximately 1 month) before receiving injections of the liver extracts. One U.S.P. unit per day of either reticulogen³ or another preparation⁴ was

³ Lilly.

⁴ Sharp and Dohme no. 2505.

injected intramuscularly. Typical growth and hemoglobin curves for 2 animals receiving folic acid and liver extract are given in figure 3.

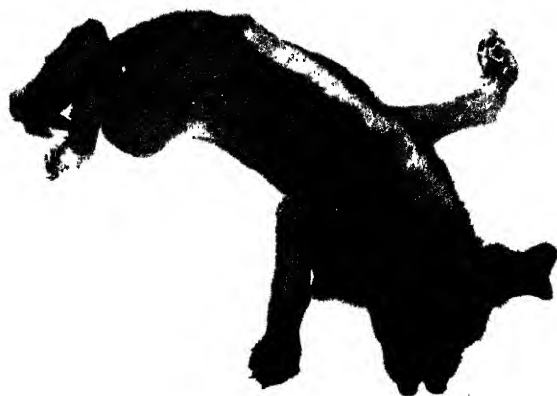


Fig. 2 A typical case of paralysis observed on a niacin-deficient diet.

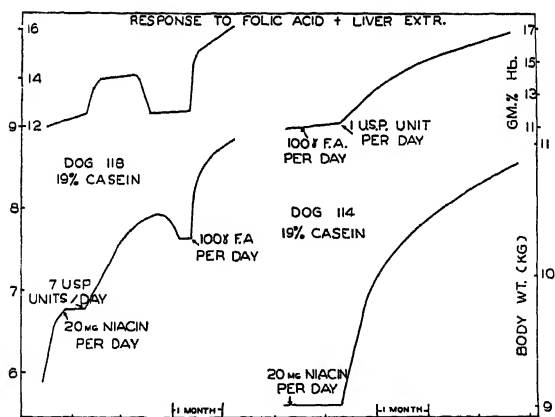


Fig. 3 Response of dogs to folic acid and liver extract.

In order to determine if folic acid was needed in this regimen, animals which had failed to respond to niacin and which were receiving adequate amounts of niacin were given comparable injections of the same liver preparations without folic acid. Some response in weight and hemoglobin occurred

(fig. 3) but these values soon returned to suboptimal levels. When folic acid was added, a complete response was seen, indicating that this factor is necessary.

When a basal ration containing 19% casein was fed, a need for folic acid could be shown. When higher levels of casein were fed, however, good responses to the pernicious anemia concentrates could be obtained without the addition of folic acid (fig. 4).

Folic acid conjugate was tried in one dog receiving the liver extract, and a response comparable to that seen in dogs receiving free folic acid was observed (fig. 3). Apparently

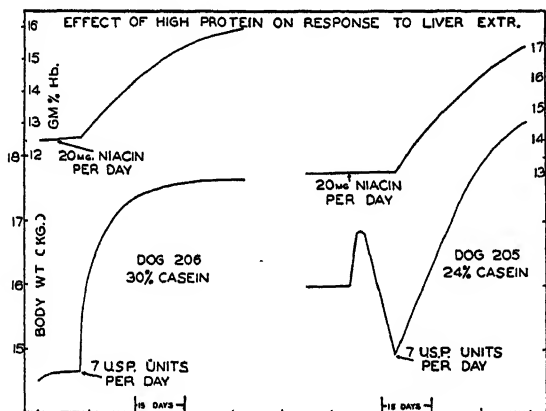


Fig. 4 Response to liver extracts of dogs receiving 24 and 30% protein rations.

the dog can utilize the bound form of folic acid, as contrasted to the monkey which, under our experimental conditions, cannot utilize the conjugated forms very effectively (Cooperman, Elvehjem, McCall and Ruegamer, '46).

Several attempts were made to develop an assay for the factor found in the liver extract. It was thought that by giving a single injection of the liver extract, a correlation between the weight or hemoglobin response and the U.S.P. unitage might be attained. It was found, however, that the animal apparently could not store the factor, since equal weight

responses were seen with the injection of several levels of the extract. Apparently only a given amount of the extract from a single injection can be utilized. In view of these results, daily injections were tried and it was found that optimal growth and hemoglobin production could be obtained with one U.S.P. unit of reticulogen injected per day. Lower levels were not tried. These results are summarized in table 2.

DISCUSSION

When a niacin-free basal ration containing 1% sulfasuxidine was fed to young growing dogs, the animals began to lose weight and developed signs of blacktongue in 14-18 days. Niacin was only partially effective in counteracting the syndrome. Folic acid helped to produce more consistent responses to niacin, but the anemia which developed did not respond to folic acid therapy. It should be pointed out that blood responses were seen in several animals receiving folic acid, but we attributed this to a failure to deplete the animals completely of the liver factor. When folic acid was withdrawn from the regimen, the animals lost weight and developed a more severe anemia. A point was reached in all cases where the animals failed to respond to the folic acid, but responded completely to the administration of liver extract.

There were many indications that we were dealing with a condition similar to pernicious anemia in the human. The anemia observed in the dog was found to be macrocytic-normochromic in nature, and it developed progressively. The animals often suffered from diarrhea and showed general lassitude. A flaccid type of paralysis was observed in several of the more advanced cases, and liver damage such as that described by Krehl et al. ('46) was also seen in some animals at autopsy.

As little as one U.S.P. unit of pernicious anemia activity was effective in bringing about a complete remission of the anemia observed in dogs which had failed to respond to niacin. In the case of reticulogen this would mean that $\frac{1}{20}$ ml

of the extract was injected per day, or that approximately 10 mg of solid material was sufficient to produce an optimal response in the dog. If we are not dealing with the pernicious anemia factor itself, then it must be something else that was concentrated to a high degree along with it.

The liver extracts were used first with adequate amounts of folic acid; when folic acid was omitted poor results were obtained with the extract. When folic acid was added, a complete response was seen, indicating that it was necessary. It would appear that all three factors — niacin, folic acid and the liver extract — are required. If any one of the three is present in inadequate amount, it is a limiting factor, and optimal growth and blood formation will not take place. This was true only when the protein level was about 19% because when the level was increased to 24 or 30% the need for folic acid could not be demonstrated. Possibly higher levels of casein favor the intestinal synthesis of folic acid in the dog.

Since the time this work was first instituted (Ruegamer et al., '47), liver extracts containing the pernicious anemia factor have been shown to contain factors which can be measured on the basis of a growth response in chickens and in rats (Bethel et al., '47; Nichol et al., '47). Between 2 and 6 weeks are required to show activity in these animals, whereas a conditioning period of approximately 3–6 months in addition to a 1–2 month test period are required for the dog. Consequently, the rat and chicken appear much more practical for the assay than the dog, providing that all three animals are responding to the same factor. However, the dog assay is still important because of the hematological changes observed. Whether or not the same factor is measured in each of the three species is still uncertain.

SUMMARY

Young growing dogs were fed a niacin-deficient purified ration containing 1% sulfasuxidine. When the animals developed symptoms of blacktongue, they were given single doses of niacin. This therapy was found to be only partially suc-

cessful in combating the loss in weight. Folic acid was found to play an important part in bringing about more consistent responses to niacin, but it had no apparent effect on the macrocytic anemia which developed progressively. Liver extracts rich in the pernicious anemia factor were effective in restoring the blood picture and general health of the animals. These extracts were only partially effective when given alone, but in combination with folic acid gave complete recovery. When higher levels of protein were fed, however (24-30%), the need for folic acid could not be shown. As little as one U.S.P. unit of reticulogen per day is sufficient to bring about complete recovery. Apparently the factor is not stored to any great degree, and must be injected regularly.

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VITAMINS REQUIRED BY SWINE FOR GROWTH, WITH SOME OBSERVATIONS ON REPRODUCTION

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The earlier literature on the adequacy of synthetic diets for pigs was reviewed by McRoberts and Hogan ('44) and requires no further comment at this time. These investigators reported that in their experience synthetic diets were inadequate for pigs. The mortality rate was high and the animals usually grew slowly, though occasionally a pig on an artificial diet would grow at almost a normal rate. The basal diet used most extensively contained thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, choline, and vitamins A, D, E, and K. The diet was not improved by the addition of ascorbic acid, biotin, inositol, and *p*-aminobenzoic acid, but it became entirely adequate for growth when a water extract of liver was included. This was regarded as evidence that the pig requires a vitamin then unrecognized.

Several groups of investigators, including Wintrobe, Stein, Follis and Humphreys ('45); Lindley and Cunha ('46); Cunha, Bustad, Ham, Cordy, McCulloch, Woods, Conner, and McGregor ('47); and Powick, Ellis, Madsen, and Dale ('47) attained a considerable degree of success in rearing pigs on synthetic diets. However, most of their experimental animals

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The data in this manuscript were taken from a thesis to be submitted by Mr. Anderson in partial fulfillment of the requirements for the A.M. degree.

were at least three weeks old at the beginning of the experimental period. Wintrobe and collaborators used a few that were 16 to 22 days old when first given a synthetic diet. One of the most successful vitamin mixtures used by this group did not contain niacin, inositol, or *p*-aminobenzoic acid. Lindley and Cunha ('46) obtained no evidence that either biotin or inositol should be included in the diet of the pig. Cunha et al. ('47) reported that their ration was not improved by the addition of folic acid and *p*-aminobenzoic acid, either alone or in combination with biotin and inositol. Powick et al. ('47) concluded that between the ages of three and 9 weeks the pig requires between 0.6 and 1.0 mg daily of nicotinic acid per kilo of live weight.

Cartwright, Wintrobe and Humphreys ('46) placed a pig at the age of 30 days on a synthetic diet which included 2% of sulfasuxidine. The pig grew slowly and developed symptoms of a nutritional deficiency. After 140 days a normocytic anemia was well established. The animal was given intramuscular injections of a commercial liver extract, presumably prepared for the treatment of pernicious anemia, and recovered. Four other pigs received this same diet, except that crude casein replaced the vitamin-free product; they were normal in appearance and their growth rates were not retarded.

Welch, Heinle, Sharpe, George and Epstein ('47) also included succinylsulfathiazole in a synthetic diet they supplied to pigs, but no evidence was obtained that it retarded the rate of growth or interfered with the production of erythrocytes or hemoglobin. After 16 days one of the pigs in the group which did not receive pteroylglutamic acid was given a chemical antagonist of that vitamin. The pig grew slowly, became unthrifty in appearance and became anemic. When the condition of the pig became critical, the vitamin-free casein in the diet was replaced by crude casein, and in addition an alcoholic extract of crude casein and human gastric juice were given together by stomach tube. After 10 days of this supplementation, the pig was returned to the original diet and

apparently became entirely normal. It is not clear why the purified diet failed to interfere with growth and hematopoiesis as described by Cartwright et al. ('46) but the difference in the vitamins supplied may have had some effect on the results. The observations of Cartwright and collaborators ('46) and of Welch et al. ('47) give some support to the conclusion of McRoberts and Hogan ('44) that the pig requires an unrecognized vitamin.

Russell, Teeri and Unna ('48) began with pigs that were 28-31 days old and maintained them as long as 469 days on a synthetic diet. The vitamin mixture did not include biotin, pteroylglutamic acid, or vitamin K. The pigs grew normally and were normal in appearance but neither the males nor females were able to reproduce. Ensminger, Bowland and Cunha ('47) observed that sows which consume synthetic diets over a long period are unable to rear their litters. Other evidences of the inadequacy of the ration were difficulty in getting the sows to conceive and abnormalities in the pigs, such as weakness at birth and a high mortality rate, hernia, kinked tails, enlarged forelegs, and abnormal liver and kidneys.

During the period since the 1944 paper of McRoberts and Hogan appeared, biotin has been supplied in larger amounts and synthetic pteroylglutamic acid has become available. The object of the present investigation has been to test the adequacy for pigs of synthetic diets containing these two vitamins, in addition to the others mentioned, from the beginning of the experimental period.

EXPERIMENTAL

The experimental procedure used has been described by McRoberts and Hogan ('44) and only its more essential features will be repeated. The new-born pigs were left with the dam for two days, as in our earlier experience the mortality rate had been high even on diets believed to be adequate if the pigs failed to receive colostrum. The diets, described in table 1, were synthetic in type but homogenized in water to

reduce the content of solids to 19%. The pigs were hand-fed from a bottle 8 times daily, at three-hour intervals, during the first two weeks. During the next 6 weeks they were fed 7 times daily, beginning at 6:00 A.M. and ending at 12:00 midnight. If kept on experiment for a longer time, the pigs were then given dry feed. Since it is essential to keep young pigs warm, they were quartered in a room held at a temperature of 75°F. or higher.

TABLE 1
Composition of experimental diets

BASAL MIXTURE				SUPPLEMENTS			
Ingredient	Diet number			Vitamins ¹ per 100 gm of diet (all diets)			
	327	330	335				
	%	%	%				
Casein				Vitamin A (I.U.)	2000	Pyridoxine-HCl (mg)	1
(Labco)	30	15	30	Vitamin D (I.U.)	400	Ca-pantothenate (mg)	3
Sucrose	30	70	30	Vitamin E (mg)	4	Nicotinic acid (mg)	4
Corn starch	5	..	5	Vitamin K (mg)	2	Choline-Cl (mg)	100
Wood pulp	..	5	5	Thiamine-Cl-HCl		Inositol (mg)	100
Lard	30	5	25	(mg)	1	Biotin (mg)	0.03
Salt				Riboflavin (mg)	1	Pteroylglutamic acid (mg)	0.2
mixture ²	5	5	5				

¹ Vitamins A and D were purchased as a concentrate from Mead Johnson and Company, Evansville, Indiana. The pteroylglutamic acid was generously supplied by Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York. All other vitamins were generously supplied by Dr. D. F. Green of Merck and Company, Inc., Rahway, New Jersey.

² Richardson and Hogan ('46).

Three separate tests of the adequacy of artificial diets were carried out. The 6 purebred Chester White pigs used in the first trial were kept on cement floors with shavings as bedding. Because of uncertainty as to the adequacy of the diet, three of the 6 received a ration which was similar to Diet 327 except that a liver extract was substituted for an equal weight of sucrose. During the first 23 days the modified ration contained 2% of a pork liver extract fraction. However, the fraction did not seem to improve the ration in any respect, and on the 24th day 5% of a water extract of pork

liver was included. Since it is doubtful that the extract had any effect on the response of the pigs, no special comment on it is required.

The other three pigs received Ration 327 with no modifications, and will be treated in more detail. As soon as they were transferred to the artificial milk they developed a diarrhea which was most marked during the first two weeks but which persisted in some degree throughout the experimental period. These symptoms were also characteristic of the two other trials which were carried out later on. It seemed impossible when the diarrhea first appeared that there could have been time for a nutritional deficiency to develop, and it seems probable now that one or more constituents of the diet is mildly irritating to the intestinal tract of a young pig. Possibly the irritation could be avoided by reducing the amount of mineral salts or by replacing sucrose with a simple sugar.

During the first three weeks the pigs grew at only a moderate rate and there was a definite, though slight, brownish exudate around the eyes of each pig, which later disappeared. After the third week the pigs had tremendous appetites and grew more rapidly than did their litter mates which had been left with the sow. It happened not infrequently that the pigs would regurgitate a small quantity of food, but this seemed to be due to excessive consumption rather than to a nutritional deficiency. Normally, young pigs feed about every two hours, or less. It was impracticable to feed the experimental diets that often, and for that reason the food intake per feeding was enormous. However, the pigs were altogether normal in appearance at the end of the experimental period and there was no definite evidence that the diets were deficient in any respect. The growth rates in all three trials are shown in table 2.

The response of the pigs in this first trial, both in rate of gain and in appearance, exceeded our expectations and was repeated with some variations in later tests.

TABLE 2
Growth of pigs on synthetic diets

NO. AND SEX	BODY WEIGHT		
	At birth	2 days old	56 days old
	lbs.	lbs.	lbs.
Trial 1, Chester Whites			
Ration 327			
38 F	3.5	4.1	40.2
15 M	2.7	3.7	35.6
19 M	2.6	3.1	32.7
Avg.	2.9	3.6	36.2
Ration 327 modified to contain liver extract:			
14 M	3.2	4.1	55.5
16 M	2.7	3.7	39.0
13 M	2.5	3.2	36.6
Avg.	2.8	3.7	43.7
Trial 2, Poland Chinas			
Ration 327, contains pteroylglutamic acid:			
4 F	3.2	3.7	27.8
3 F	3.0	3.4	21.6
1 F	3.0	3.5	22.8
Avg.	3.1	3.5	24.1
Ration 327, does not contain pteroylglutamic acid:			
8 F	2.6	3.5	18.5
1 M	3.0	3.5	18.7
5 F	2.7	3.3	25.0
Avg.	2.8	3.4	20.7
Trial 3, Duroc Jerseys			
Ration 327, contains pteroylglutamic acid:			
On screen floors			
61 F	3.6	3.9	29.5
58 M	3.1	4.0	29.3
Avg.	3.4	4.0	29.4
On cement floors			
60 F	3.8	4.0	34.0
55 M	2.2	3.5	17.0
Avg.	3.0	3.8	25.5
Ration 334, does not contain pteroylglutamic acid:			
On screen floors			
64 F	3.6	3.7	23.5
59 M	3.4	4.1	35.2
Avg.	3.5	3.9	29.9
On cement floors			
56 F	2.9	3.6	36.0
60 M	4.4	4.3	33.6
Avg.	3.7	4.0	34.8
Fortified cow's milk:			
On cement floors			
57 F	2.7	3.4	56.5
63 M	4.4	4.9	54.0
Avg.	3.6	4.2	55.3

In the next trial the pigs were purebred Spotted Poland Chinas. They were on floors of wide mesh screen, in order to reduce the possibility of coprophagy. An attempt was also made in this trial to determine whether pteroylglutamic acid is required by the pig and the animals were, therefore, divided into two groups. One received Ration 327 and the other Ration 334, which does not contain pteroylglutamic acid but is identical with Ration 327 in all other respects.

The outcome of the second trial was less encouraging than that of the first. The diarrhea was not more pronounced than in the first trial and the pigs were normal in appearance, but the rate of growth was disappointing. The explanation of the difference is not apparent, but several possibilities have been considered: (1) The pigs may have been of a slow-growing strain. (2) The wire screens may have been responsible for the retardation by reducing the practice of coprophagy. (3) The trial was conducted in the winter months and during that season the heating system was unable to keep the room as warm as was desired. The pigs which received pteroylglutamic acid grew more rapidly than those which did not receive it, but comparison with trial 3 suggests that the difference was fortuitous.

There were 10 pigs in all in the third trial. Two were used as positive controls to establish the growth rate on an adequate diet. These two pigs received fortified cow's milk (1 liter of milk plus 60 gm sucrose, 2.5 gm ferrous sulphate, 0.2 gm cupric sulphate, 0.2 gm manganous sulphate, 0.02 gm potassium iodide), and were on cement floors with shavings. There were 8 experimental pigs; 4 were on floors of wire screen and 4 on cement floors with shavings for bedding. This arrangement should show whether or not coprophagy had any relation to the nutritional status of the pigs. Two of each group received Ration 327 and two received Ration 334, in order to obtain additional data on the requirement of the pig for pteroylglutamic acid. The pigs were purebred Duroc Jerseys. They did not grow as rapidly as did those in the first trial, but more rapidly than those in the second.

There were intermittent attacks of diarrhea, but the pigs had the appearance of health and thrift and seemed to be normal in every respect. Insufficient data are available for a final decision, but the evidence indicates that when pigs consume artificial, so-called "synthetic" diets, they grow more rapidly when on solid floors. It is difficult to eliminate all possibility of access to feces, but it seems quite certain that pigs can attain an average rate of growth without practicing coprophagy. The pigs not receiving pteroylglutamic acid grew as rapidly as did those which did, and we have no reason now to suppose that this vitamin is a dietary essential for the pig.

The only animal which calls for special comment is 55M, which was quartered on the cement floor and received pteroylglutamic acid. From the beginning this animal seemed eager for food but would never consume at any one time as much as the others did. The pig seemed definitely abnormal in some way, but there was no specific symptom which suggested that the abnormality was due to inadequacy of the ration. When 60 days of age, with a weight of 19.8 pounds, this pig was changed to the diet of fortified cow's milk, which was supplied in an open container so the milk was available practically all the time. At the end of 25 days the pig weighed 43 pounds. While consuming the synthetic diet the average daily gain was 0.23 pounds, and after the change to fortified cow's milk, the average daily gain was 0.93 pounds, a normal rate for that age. When 85 days old, this pig was given a practical ration but it consumed little feed and did not gain in weight. The animal was sacrificed for post-mortem examination² but the only abnormalities observed were inflammation in restricted areas of the colon and petechial areas in the stomach with some deep-seated fibrosis, which were presumably the consequence of a previous low-grade inflammation. It is impossible to decide whether or not the diet of the pig was responsible for its lack of thrift.

The pigs which consumed cow's milk grew much more rapidly than did those fed the "synthetic" milk, and we have

² Courtesy of J. E. Weinman, D.V.M.

adopted the working hypothesis that the artificial diet is deficient in a nutrient essential for maximum growth and for the optimum nutritional state.

During the first trial, in the fall of 1946, there was one female pig on Ration 327 and since this group had exceeded all expectations in appearance and performance, an attempt was made to continue her on an artificial diet through a reproductive period. When she was 87 days old, her ration was modified somewhat to reduce the protein content. The composition of this ration, no. 330, is shown in table 1. This gilt, no. 38, continued to grow at a tremendous rate, and eventually her feed intake was limited to prevent her from becoming overfat. She conceived on March 12, and farrowed on July 7, with no untoward incidents. She gave birth to 11 pigs that were alive and two that were dead. One of the dead pigs had died at least two or three weeks before parturition; the other was smothered in the chorionic membrane. The appearance of the pigs would indicate that the milk of this sow was adequate in quality, but never adequate in quantity. Five of the mammary glands were inflamed and nonfunctional, and only 5 of the others secreted significant quantities of milk. There was delay in the initiation of milk secretion and at the beginning of the second week only 5 pigs were alive. During the first 10 days of lactation the feed intake was low and therefore the ration was changed on the 11th day to no. 335, table 1. After that time the feed intake increased and apparently the milk flow increased also. When the litter was about 5 weeks old the rate of feed consumption declined again, with a parallel decrease in the rate of milk secretion, and it was also noted that the sow was severely anemic. In view of the report of Cartwright, Wintrobe, and Humphreys ('46), the sow was given injections of 2 ml (30 units) of liver extract³ for 14 successive days, but the treatment was ineffective. Whether it was given too late, or whether it did not contain the nutrients she required, we are unable to say. She died on the 56th day

³ Eli Lilly Co., Indianapolis, Indiana.

TABLE 3

Abbreviated history of the sow reared on an artificial, so-called "synthetic" diet

AGE	WEIGHT	DAILY GAIN IN WEIGHT	DAILY FEED INTAKE	ERYTHROCYTE COUNT
<i>days</i>	<i>lb.</i>	<i>lb.</i>	<i>lb.</i>	<i>million/mm³</i>
Ration 327				
56	40			7.485
86	63	0.76	1.6	
Ration 330				
116	119	1.86	3.8	7.625
146	182	2.10	6.6	
176	246	2.13	6.8	
206	306	2.00	6.3	
236	352	1.53	4.5	7.526
266	410	1.93	5.1	
294	465	1.96	6.1	
Farrowed on 294th day				
295	419		0.0	
306	369		0.5	7.724
310	369		4.0	
Ration 335				
320	352		6.0	
330	348		8.0	
334	338		7.0	3.040
337	330		4.0	1.225
339	327		1.0	2.375
346				1.870
350	died			

after farrowing.⁴ A brief history of this animal is shown in table 3.

When it became evident that sow 38 was anemic her 5 remaining pigs were removed, and reared on the same diet as their mother had received. The average weight at 56 days was 24 pounds, with a range of 16.7 to 34.5 pounds. This was a

⁴ Pathologist's report on sow 38 (courtesy of M. P. Neal, M.D.): *Bone marrow*: no recognizable hematopoietic tissue; functional marrow replaced by non-functional fat; *liver*: remarkably pronounced interlobular connective tissue; degeneration and necrosis of liver cells beginning around the central veins and extending through the central zone area; *kidneys*: extensive albuminous degeneration and hydropic infiltration of the proximal tubules; *spleen*: a loss of Malpighian corpuscles and of parenchymatous elements and a deposit of fibrinous material in a tissue closely resembling hematopoietic structures.

remarkable recovery, and indicates that the diet could not be seriously inadequate for growth.

DISCUSSION

It is clear that our efforts to rear pigs on synthetic diets were much more successful than those of McRoberts and Hogan ('44). The reason is uncertain, but some of the possibilities will be mentioned. The salt mixtures used in the two trials were not the same, but neither the qualitative nor quantitative differences seemed large enough to be significant. There were minor differences in the amounts of the vitamins included in the diets, but according to present knowledge the least amount of any of the vitamins supplied by McRoberts and Hogan was more than enough. It seems improbable that pteroylglutamic acid contributed to our greater success in these recent trials, because the pigs grew as well without it as they did with it. Biotin and inositol were always included in the rations described in this report but our experience, and that of other investigators mentioned, leaves it uncertain whether either of these vitamins contributed to the improvement in the diet. The average growth rate of the experimental pigs was equal to that of suckling pigs in commercial production, but the high degree of variability requires some comment. The variable growth rates may be partially explained by genetic differences, but it seems more probable that the experimental diet was inadequate in some degree. This conclusion is supported by the poor appetite of sow 38 after farrowing, her failure in milk secretion, her severe anemia, the extreme pathological condition of some of her internal organs, and her early death. However, the possibility that some or all of these abnormalities were the consequences of mastitis may require consideration.

SUMMARY

1. Seventeen pigs were reared on a synthetic diet from the time they were two days old until they were 56 days, or more, of age. There were no mortalities, though all of the pigs were

subject to intermittent attacks of diarrhea. The rate of growth was variable but the average weight at 56 days was as high as would be expected according to current growth standards. With one exception, all of the pigs were normal in appearance.

2. One female pig was retained on the synthetic diet through the reproductive stage. She bore a normal litter but her flow of milk was scanty. She became severely anemic while lactating, and died. On autopsy an extreme pathological condition was discovered in the bone marrow, liver, kidneys and spleen.

3. These findings indicate that the artificial, so-called "synthetic" diet used in this study is slightly inadequate for growth and seriously inadequate for lactation.

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SOUTHERN PEAS AND OTHER LEGUME SEEDS AS A SOURCE OF PROTEIN FOR THE GROWTH OF RATS

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THREE FIGURES

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Southern peas (edible varieties of the cowpea, *Vigna Sinensis*, Brittingham, '46), of which the most generally consumed variety is the Blackeye pea, are an important source of food for a large section of the population throughout the South. Comparatively few studies have been carried out on the nutritive value of varieties of Southern peas, and since they are used widely as a source of human food it is desirable to have more information about their relative value as a source of protein for growth.

Osborne, Mendel and Ferry ('12) found the isolated globulin, virginin — the chief protein of the cowpea — to have a slightly greater nutritive value than wheat gliadin. Finks, Jones and Johns ('22) in a study of the field varieties, Groit and Brabham, obtained a growth rate in rats of $\frac{1}{3}$ to $\frac{2}{3}$ of normal with either raw or cooked cowpea meal. These same authors found that the addition of 0.33% cystine to the cooked meal gave a normal rate of growth, but the addition of the cystine to the raw meal gave no better growth than the raw meal alone.

An increase in the nutritive value of soybean protein on heating has been demonstrated by several investigators, and

these studies have been reviewed recently by Evans and McGinnis ('46). Finks and Johns ('21) reported that rats fed raw Lima beans, *Phaseolus lunatus*, failed to gain weight even when the protein was supplemented with 0.3% of cystine. The cooked beans were no better than the raw beans unless supplemented with cystine. When 0.3% of cystine was added to the cooked beans, the rats grew at a normal rate.

Since cystine is not an indispensable amino acid, the response to the addition of cystine in these earlier experiments requires some explanation. Rose ('38) has shown that cystine stimulates growth when methionine is supplied in sub-optimal quantities, but not when it is entirely absent from the diet. The diets which were used in these earlier experiments must have contained a sub-optimal amount of methionine, and therefore growth was stimulated when cystine was added.

Everson and Heckert ('44) tested the nutritive value of several legumes for the growth of rats, both in the raw state and after heating in a pressure cooker at 15 pounds for 15 minutes. The rats which received raw kidney beans, navy beans or pinto beans lost weight and died within 3 or 4 weeks. Those which received raw Lima beans lost weight but survived the 8-week experimental period. The nutritive value of all the legumes was increased by heating with the exception of peas in which it was decreased slightly. Woods, Beeson and Bolin ('43) and Lehrer, Woods and Beeson ('47), in a study of the nutritive value of Alaska field peas, reported that the daily gain was tripled and the protein efficiency doubled when either the cooked or raw peas were supplemented with 0.3% of methionine. Heating the peas dry for 1½ hours at 140°C. or autoclaving for 1½ hours at 17 pounds pressure decreased the nutritive value, and this decrease was attributed to the effect of heat on the protein. Russell et al. ('46) reported the results of their studies on several varieties each of Lima beans, snap beans (*Phaseolus vulgaris*) and English peas. The legumes were soaked overnight and cooked in the soaking water in a procedure similar to that used for human consumption. Growth was poor in all cases unless the legumes

were supplemented with 0.1% of methionine. Raising the level of methionine to 0.6% gave a further growth response except for three varieties of peas, in which cases a loss in weight occurred.

Ham and Sandstedt ('44) and Ham et al. ('45) reported the presence of a trypsin inhibitor in soybean meal, and Bowman ('44) found the trypsin inhibitor in navy beans as well as soybeans. Kunitz ('45, '46, '47) has isolated the crystalline trypsin inhibitor from soybeans and has studied its properties. This inhibitor is destroyed by heat. It is probably present in certain other legume seeds, but available data indicate that it is not present to any appreciable extent in the various varieties of Southern and English peas which were used in this study.

Observations on the comparative nutritive values of Southern peas (*Vigna Sinensis*), Lima beans (*Phaseolus lunatus*), pinto beans (*Phaseolus vulgaris*), English peas (*Pisum sativum*), casein and egg albumin are given in this report.

EXPERIMENTAL

Albino rats 28 days old and weighing 35 to 45 gm were given the experimental diets for a period of 4 weeks. Litter mates were matched as to sex and weight and distributed as far as possible among the groups which received the different legumes. They were kept in individual cages and food and water were supplied ad libitum except in the paired feeding trials. Two separate groups, consisting of two males and two females each, were used in every test and the two tests were run at different times. Each curve in the charts represents the combined average gain in weight of the 8 rats used in the test.

The legumes were purchased from a seed supply house or grown on the Texas A. and M. Horticultural Farm. The nutritive value of both the raw and heated legumes was studied. Before heating, 1 kilo of the ground seed was mixed with 1500 ml of distilled water and allowed to stand for one to two hours. The mixture was then heated in the autoclave for 30 minutes at 15 pounds pressure and dried in an air oven at 60°C. The

legumes were added to the diet at a level equivalent to 10% protein ($N \times 6.25$) in all cases except when comparison was made of casein with Southern peas by the ad libitum and paired feeding methods; the amounts provided in these tests are given in figure 1. The casein and egg albumin were added at the levels shown in figures 1 and 3. The composition of a typical diet which supplies 10% protein is given in table 1.

Ad libitum and paired feeding methods

A preliminary test was carried out to determine whether or not the ad libitum method of feeding would be a satisfactory

TABLE 1
Composition of a typical diet

BASAL MIXTURE	%	VITAMIN SUPPLEMENTS PER 100 GM OF BASAL MIXTURE	
Legume (equivalent to 10% protein)	44	Menadione	mg 2.5
Cerelose	38	Alpha tocopherol	2.5
Wood pulp	3	Thiamine hydrochloride	0.4
Mineral mixture ¹	5	Riboflavin	0.4
Lard	10	Pyridoxine	0.4
VITAMIN SUPPLEMENTS PER 100 GM OF BASAL MIXTURE		Calcium pantothenate	1.0
		Niacin	5.0
Vitamin A	3,000 I.U. ²	Choline chloride	100.0
Vitamin D	425 I.U.	Biotin	0.01

¹ Richardson and Hogan, '46.

² Vitamins A and D were supplied by Mead Johnson's Oleum Percomorphum.

means of investigating the relative value of two proteins if they were fed at the same protein level. In this test Southern peas were compared with casein by both the ad libitum and the paired feeding methods. Both Southern peas and the casein were supplied at protein levels of 12 and 15%. These data are summarized in figure 1. The difference in weight gains are practically the same regardless of whether the rats were fed by the paired feeding or the ad libitum method.

This observation shows that the calculation of protein efficiency does not give any additional information in regard to

the relative value of the two proteins. Hegsted and Worcester ('47) have concluded, from a study of the relation between protein efficiency and gain in weight on diets of constant protein content, that gain in weight alone and protein efficiency alone are equally accurate in measuring the relative value of proteins. In view of these observations, the rats in all subsequent experiments were fed ad libitum and the curves in the charts represent the average gains in weight obtained by this method.

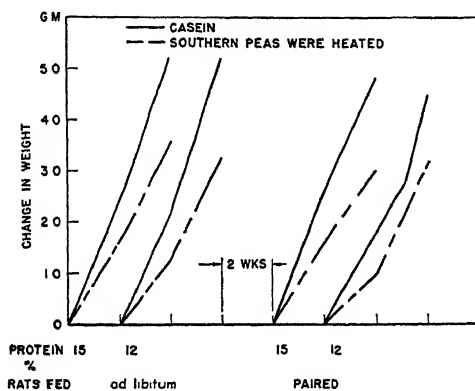


Fig. 1 Comparison of the average gain in weight of rats when they were fed Southern peas and casein by the ad libitum and by the paired feeding methods.

Amounts of methionine required

The effect of supplementing heated pinto beans and Southern peas with two levels of methionine is summarized in figure 2. Either legume supports a very slow rate of growth when it is fed as the sole source of protein in the diet, but when supplemented with 0.2% methionine practically normal growth is obtained. Two-tenths % of methionine gave a maximum rate of growth. One-tenth % was not enough, and in the few trials where 0.3% was fed the gains were no faster than they were with 0.2%.

Comparison of legumes, legumes plus methionine, casein and egg albumin as sources of protein

Southern peas were compared with Lima beans, pinto beans, English peas, casein and egg albumin as sources of protein for the growth of rats. The results are summarized in figure 3.

Rats which received raw Southern or raw English peas grew slowly, but they grew at a slightly faster rate than those which received the heated peas. When the English peas were supplemented with 0.2% methionine the rats grew at a normal rate, but again those receiving raw peas grew at a slightly faster rate than those receiving heated peas. A slight de-

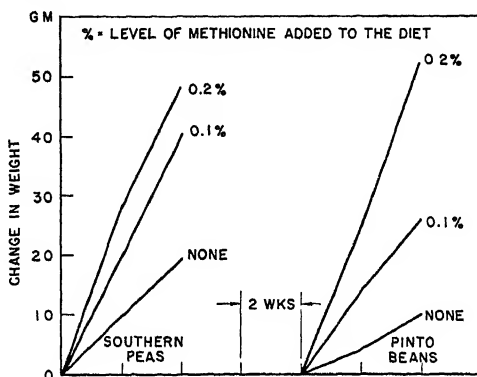


Fig. 2 The amount of methionine required to give a maximum gain in weight in rats when all the protein in the diet is supplied by pinto beans or Southern peas.

crease in the nutritive value of peas when autoclaved or heated dry has been reported also by Everson and Heckert ('44) and by Lehrer et al. ('47). Supplementing the heated Southern peas with 0.2% methionine increased their nutritive value, but the average gain was less than it was with egg albumin or with the other legumes plus methionine.

The results obtained with raw Lima and raw pinto beans differed from those which were obtained with either of the raw peas. The rats which received the raw beans lost weight and two receiving raw Lima beans died on the 23rd and 26th day, respectively. The weight for this group for the 4th week in

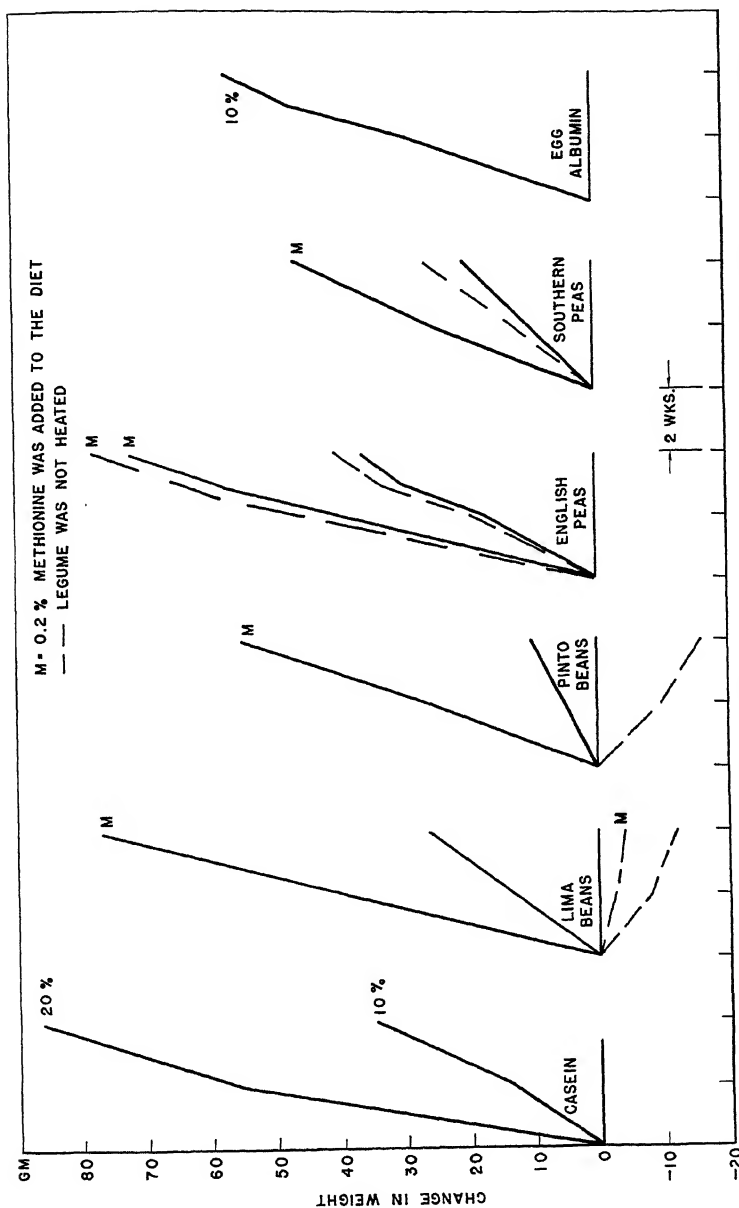


Fig. 3 Comparison of casein, Lima beans, pinto beans, English peas, Southern peas and egg albumin as sources of protein for the growth of rats. The varieties of legumes were as follows: Lima bean, Henderson bush; pinto bean, commercial; English peas, Little Marvel; Southern peas, Dwarf California Blackeye no. 5.

figure 3 represents the average of 6 rats. The addition of 0.2% methionine to the raw Lima beans decreased the rate loss slightly, but these beans were a very poor source of protein for the growth of rats even with methionine added. This suggests that the low nutritive value of the raw Lima bean is probably due to the presence of a trypsin inhibitor as well as an inadequate amount of methionine.

English peas were superior in nutritive value to heated Lima beans and to raw or heated Southern peas, and the heated Lima beans were slightly superior to Southern peas. The protein of heated pinto beans had a lower value than the protein of any of the other legumes studied until it was supplemented with methionine. When the heated legumes were supplemented with 0.2% methionine the rats grew at a rapid rate in every case, but the greatest gains were obtained with Lima beans and English peas. The rats receiving these legumes grew faster in both cases than those which received egg albumin. Pinto beans plus methionine supported as rapid a rate of growth as egg albumin but the Southern peas, although superior to 10% of casein, were inferior to egg albumin.

Amino acid deficiency of Southern pea protein

The data which were discussed in the preceding section show that in Southern pea protein methionine is either low or unavailable, and suggest that there is either a deficiency or a low availability of one or more other essential amino acids. Schweigert ('48) has shown that all the tryptophane in soybean oil meals is not available for the growth of chicks, and Kuiken and Lyman ('48) have reported that the lysine in cottonseed flours is only 64.0% available, while some of the other essential amino acids are 95 to 100% available. Tests were carried out with these two amino acids to determine if they were limiting factors in Southern pea protein. The results are summarized in table 2.

The rats which received Southern peas supplemented with methionine alone gained less than those which received lysine

and tryptophane in addition to methionine. A statistical analysis showed that these differences are highly significant. The gains of the rats which received Southern peas supplemented with the three amino acids did not differ significantly from those of the animals receiving egg albumin. It may be concluded from these data that Southern pea protein supplies an inadequate amount of lysine or tryptophane or both for the normal growth of rats. It has not been demonstrated whether the deficiency is due to a low amino acid content or to a low availability of the amino acid or acids. Lysine and tryptophane were not tested separately.

TABLE 2

Amino acids as supplements to Southern pea protein for the growth of rats

SOURCE OF PROTEIN	AMINO ACID SUPPLEMENT		RATS	AVERAGE GAIN IN WEIGHT ¹
	%		no.	gm
Southern peas	Methionine	0.2	10	44
Southern peas	Methionine	0.2	12	57
	Lysine	0.5		
	Tryptophane	0.1		
Egg albumin	None		12	56

¹ Experimental period 4 weeks.

Comparison of three varieties of Southern peas

The three varieties of Southern peas which are generally grown for human consumption in Texas are Long Pod Cream, Dwarf California Blackeye no. 5 and Jackson Purple Hull. These varieties were compared as sources of protein for the growth of rats at a level equivalent to 10% of protein. The results are summarized in table 3. Each variety was autoclaved and tested with and without methionine. The rats gained more in each case when methionine was added. The Long Pod Cream and California Blackeye no. 5 varieties were about equal in value when they were supplied at the same level of protein. However, it requires a total of 42% of the California no. 5 peas as compared to 37.5% of the Long Pod

Cream peas to give the same level of protein in the diet. The rats which received Jackson Purple Hull peas gained an average of 12 gm without and 32 gm with methionine; those which received Dwarf California Blackeye no. 5 and Long Pod Cream each gained an average of 20 gm without methionine and 45 gm with it. A statistical analysis showed that the differences in the gains of the rats which received the Jackson Purple Hull peas and those which received the other two varieties were highly significant, and it may be concluded that these peas have a lower nutritive value than the other two varieties when fed either with or without methionine. The data show that the protein of the Jackson Purple Hull variety

TABLE 3

Comparison of the nutritive value of the protein of 3 varieties of Southern peas

VARIETY	PROTEIN CONTENT	PEAS REQUIRED TO GIVE 10% PROTEIN	AVERAGE GAIN IN WEIGHT ¹	
			None	Methionine 0.2%
	%	%	gm	gm
Jackson Purple Hull	27.0	37.0	12	32
Dwarf Calif. B. no. 5	23.8	42.0	21	45
Long Pod Cream	26.7	37.5	20	45

¹ Experimental period 4 weeks. There were 2 trials in each test consisting of 6 rats each. The average gain in weight is the average of 12 rats in each case.

differs from that of the other varieties either in amount or in the availability of some other indispensable amino acid or acids.

SUMMARY

Lima beans, pinto peas, English peas and Southern peas were compared with casein and with egg albumin as sources of protein for the growth of rats. The legumes were tested raw and after they had been autoclaved at 15 pounds pressure for 30 minutes. The heated Lima and pinto beans were superior to the raw beans as sources of protein; the raw Southern and English peas were slightly superior to the heated peas.

The nutritive values of all the legumes were increased by the addition of 0.2% of methionine if the legume was heated before the methionine was added. Raw Lima beans were improved only slightly by the addition of methionine. On the other hand, raw English peas were improved markedly by supplementation with this amino acid, and the raw peas plus methionine were slightly superior to egg albumin when the heated legumes were supplemented with 0.2% of methionine. Southern peas were superior to casein but they had a lower value than egg albumin unless lysine and tryptophane were fed in addition to methionine.

In tests with Southern peas and with pinto beans 0.2% of methionine was required to give a maximum rate of growth. One-tenth % was not enough and 0.3% was no better than 0.2%.

The Jackson Purple Hull, Long Pod Cream and Dwarf California Blackeye no. 5 varieties of Southern peas were compared with each other as sources of protein for growth. The tests were carried out in each case with heated peas and with and without the addition of methionine. The Jackson Purple Hull had a lower nutritive value in both tests than the other two varieties. These data suggest that protein availability may be lower in the Jackson Purple Hull, or that a smaller quantity of one or more of the essential amino acids may be present.

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THE OCCURRENCE OF 5 B-VITAMINS
IN THE TISSUES OF PREGNANT RATS FED
RATIONS SATISFACTORY AND
UNSATISFACTORY FOR
REPRODUCTION ¹

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Unsuccessful reproduction in rats, as evidenced by the incidence of resorptions, the development on the day of parturition of a syndrome resembling toxemia of pregnancy, and the production of many non-viable young, has been observed in animals fed a ration containing partially dried autoclaved pork muscle and yeast as the principal sources of the B-vitamins and of protein (Swanson et al., '43; Armstrong and Swanson, '43). The toxemia characteristic of animals fed this experimental ration developed suddenly; the rats lost all muscle tone, the hair was erect, the feet and ears were very pale and the entire body felt cold. Hematuria and vaginal hemorrhage frequently were present. Death was preceded by convulsions or coma. In most cases the female died before parturition occurred, although in some instances the young were born dead. The syndrome of toxemia occur-

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red sporadically, varying from a maximum incidence of 43% of the animals during initial studies to less than 5% during certain years. The number of pregnancies terminating in resorptions varied from zero to 11% in the earlier studies. More recently the ratio has been somewhat greater.

For the past several years this ration has been studied in an effort to determine the nature of the dietary deficiency responsible for the poor reproductive performance. The diet was supplemented in turn with various materials, some natural and others synthetic. Fresh beef liver alone afforded protection against the reproductive disorders. Beef muscle, when substituted for the pork in twice the quantity in which it occurred in the original diet also improved the reproductive record. In general, however, little conclusive evidence was obtained as to the dietary causes of unsuccessful reproduction, and a new method of attack was necessary.

It seemed that if the problem could be approached from the standpoint of examining vitamin depositions in tissues both of normal animals and rats fed the experimental pork diet, information might be gained regarding the nature of the dietary inadequacy. The facts (a) that liver exerted a protective influence, and (b) that the recent occasional appearance of the toxemia occurred when yeasts containing higher quantities of certain of the B vitamins were used, led us to believe that a deficiency, either acute or border-line, of one or more of the B-vitamins might be the causative factor.

The present study reports data on the occurrence of thiamine, riboflavin, niacin, pantothenic acid and biotin in the hepatic, carcass, and fetal tissues of experimental and control groups of rats at the termination of the first and second pregnancies. One group received the original pork-containing ration designated as Pork I and the second, a modification of the Steenbock stock diet (Steenbock XV) which has been used successfully in this laboratory for many years for the production of stock animals.

EXPERIMENTAL

Animals

Seventy-two female rats of inbred Wistar stock were used in the experiment. All animals received a modification of the stock ration originally formulated by Steenbock ('23) until the time of the opening of the vaginal orifice. The distribution of the experimental animals is given in table 1. When animals receiving the Pork I ration developed the syndrome of toxemia, they were studied as a separate group. Replacements were made from stock so that a total of 10 females comprised each main experimental group.

TABLE 1
Distribution of experimental animals

GROUP	DIET	NUMBER OF ANIMALS	TIME AT WHICH ANIMALS WERE SACRIFICED
1	Steenbock	10	Day of opening of vaginal orifice
2a	Steenbock XV	10	28 days (± 3 days) after opening of vaginal orifice
2b	Pork I	10	28 days (± 3 days) after opening of vaginal orifice
3a	Steenbock XV	10	21.5 days (± 4 hours) after 1st mating
3b	Pork I	10	21.5 days (± 4 hours) after 1st mating
4a	Steenbock XV	10	21.5 days (± 4 hours) after 2nd mating
4b	Pork I	10	21.5 days (± 4 hours) after 2nd mating

Four animals which developed toxemia were available for assay. Two of these females were originally in group 4b, and two were taken from other experiments. The latter animals had been receiving a ration similar to Pork I except that the amount of yeast had been reduced from 5 to three parts. The syndrome of toxic pregnancy appeared earlier in the gestation period during this study than was the case in previous investigations; 3 of the 4 animals showed pronounced signs of toxemia on the 18th day of pregnancy and one on the 16th day. Tissues were taken for analysis directly following the death of one animal. The other three were in a succumbant state.

Diets

Both rations were offered ad libitum and records of the daily food consumption were kept. The Pork I ration contained the following ingredients: partially dehydrated pork muscle, 25 gm²; cornstarch, 53 gm; yeast³, 5 gm; agar-agar, 2 gm; sodium chloride, 1 gm; Osborne and Mendel salt mixture, 4 gm; butterfat, 8 gm; and cod liver oil, 2 gm. The pork muscle from fresh defatted hams was ground, canned in tin, and autoclaved for 65 minutes at 15 pounds pressure. Before the pork was incorporated into the ration it was spread on shallow Monel metal trays and dried to $\frac{1}{2}$ its original weight at a temperature that did not exceed 180°F. Approximately one hour was required. The ingredients of the ration were mixed for 25 minutes in a Hobart mixer, after which the ration was refrigerated. A quantity of diet sufficient to meet the needs of the experimental group for three days only was prepared at any one time. Similarly, any ration remaining in the food cups after 24 hours was weighed and discarded.

Since resorptions were appearing more frequently among the animals fed the Pork I ration than had been the case in earlier studies, 1 mg of alpha-tocopherol acetate was given orally for the first 10 days of pregnancy to rule out any criticism that the animals were not receiving enough tocopherol to meet the needs of normal reproduction.

The modified Steenbock diet was composed of the following items: yellow cornmeal, 56 gm; crude casein, 5 gm; linseed meal, 16 gm; ground alfalfa, 2 gm; sodium chloride, 0.5 gm; calcium carbonate, 0.5 gm; yeast⁴, 9.5 gm; yeast (irradiated), 0.5 gm; and wheat germ, 10 gm. To each 100 gm of the dry

²Equivalent to 15 parts protein.

³Northwestern Yeast Co., Chicago, Ill.

⁴Pabst IV.

ration⁵ were added 33.3 gm of dried whole milk⁶. The ration was further fortified with 5 gm of ground round of beef⁷ and 10 gm of fresh carrot⁸, each fed three times weekly. Fifty mg of cod liver oil were fed daily in a supplement cup.

Mating of animals

The animals in groups 3 and 4 were mated when they were 10 to 11 weeks of age. Cells from the vagina were examined daily thereafter until pregnancy ensued. Positive matings were established by identification of sperm in the vagina or by the presence of a vaginal plug. The animals were sacrificed as near the termination of pregnancy as possible.

Numerous observations in earlier studies had shown that females fed the Pork I diet rarely weaned their young, while mothers receiving the Steenbock XV ration almost always were able to do so. By eliminating the first litters born to females in groups 4a and 4b, it was possible to evaluate the two rations as to adequacy for reproduction alone. These females were remated immediately for a second pregnancy.

Preparation of samples for analysis

The animals were killed by injection of sodium pentobarbital. The hair and skin were removed as rapidly as possible and these tissues, together with the intestinal tract and its contents, were weighed and discarded. The liver was excised and weighed, and the entire organ mixed in a Waring Blender with acetate buffer (pH 4.6-4.8). The suspension was diluted with distilled water to a final weight of approximately 250 gm.

⁵ The trace elements potassium iodide, manganese sulfate, potassium aluminum sulfate and copper sulfate were added to the dry basal ration.

⁶ Klim. This amount was equivalent to approximately 25 ml of reconstituted milk and was the amount offered routinely to all pregnant stock animals in this laboratory.

⁷ Ground round of beef purchased in advance and stored at 20° F.

⁸ Fresh carrots purchased in advance and stored at 20° F.

In the case of the pregnant animals, the fetuses were removed from the uterus, weighed, and carefully examined. The total fetal tissue was blended in the Waring Blendor.

Carcass weight was determined by difference and was equal to the total weight of the animal minus the weight of the hair, skin, intestinal tract, liver, and in certain cases the fetuses. The carcass was ground in a meat grinder two or three times, after which the entire tissue was transferred to a Waring Blendor for more thorough mixing. All tissues were transferred to $\frac{1}{2}$ pint Kerr jars, covered with a thin layer of

TABLE 2
Average daily intake of vitamins of rats during pregnancy

VITAMIN	VITAMIN INTAKE			
	Steenbock XV		Pork I	
	1st pregnancy	2nd pregnancy	1st pregnancy	2nd pregnancy
	μg	μg	μg	μg
Riboflavin	90	87	36	37
Niacin	787	699	528	554
Biotin	2.71	2.69	0.38	0.40
Pantothenic acid	1088	1047	131	137
Thiamine	145	140	130	136

toluene and frozen at -10°F . The samples were stored in the frozen state until analyses could be made.

Analytical procedure

The tissues, rations, beef, and carrots were prepared for the various vitamin assays as follows: riboflavin, hydrolysis with 0.1N H_2SO_4 ; niacin, hydrolysis with 1N H_2SO_4 ; biotin, hydrolysis with 4N H_2SO_4 for two hours; pantothenic acid, enzymatic digestion at pH 4.6 with Mylase P; and thiamine, digestion with a combination of takadiastase and papain. The microbiological method was used for all assays except thiamine, which was determined by the Hennessy method ('42). The basal medium used was that of Landy and Dicken ('42).

RESULTS AND DISCUSSION

Vitamin intake

The respective quantities of each vitamin consumed by the experimental groups during the first and second pregnancies are given in table 2. The Pork I diet provided markedly less pantothenic acid and biotin, somewhat less riboflavin and niacin, and approximately the same amount of thiamine as was furnished by the stock ration. It should be mentioned that the amount of food consumed by the 10 females of a given group agreed closely, and that there was very little change in total food consumed as pregnancy progressed.

TABLE 3

Average weight of hepatic, "carcass," and fetal tissues

GROUP	LIVER	"CARCASS"	NUMBER OF YOUNG PER LITTER	FETUSES
	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
3a Steenbock XV	10.0	143.9	8.6	34.7
4a Steenbock XV	10.3	160.2	10.3	42.5
3b Pork I	9.7	146.4	4.7	17.8
4b Pork I	9.5	162.0	7.7	28.8
"Toxic" animals	7.2	150.9	11.7	3.6

General observations

The animals fed the two experimental rations grew at the same rate in the 4-week interval preceding pregnancy, the average weight gains of each group being 76 and 78 gm. Variation was observed, however, in the response of the animals to the two dietary regimens during pregnancy. The two litters produced by females receiving the Steenbock XV ration looked normal in every respect.

The reproductive performance of females fed the Pork I diet was unsatisfactory. Two of the 20 females developed the pregnancy disorder resembling toxemia, several animals resorbed their young, and the number of young borne by the females of groups 3b and 4b averaged only 4.7 and 7.7.

Observations relating to the respective weights of the maternal carcass, the maternal liver and the fetuses produced are shown in table 3. The small size of the livers of the toxic animals is characteristic (Armstrong, '39). All livers of rats fed the pork-containing diet were mottled, yellow, and somewhat friable.

TABLE 4
Average concentration of vitamins in the tissues of rats at parturition

VITAMIN	DAILY VITAMIN INTAKE	RATION	PREGNANCY	LIVER	"CARCASS"	FETUSES
	μg			$\mu g/gm$	$\mu g/gm$	$\mu g/gm$
Riboflavin	90	Steenbock XV	1	21.4	2.4	2.6
	87	Steenbock XV	2	24.3	2.3	2.5
	36	Pork I	1	18.4	2.3	1.9
	37	Pork I	2	19.7	2.3	1.8
		"Toxic" animals	—	27.0	2.1	1.5
Niacin	787	Steenbock XV	1	112.9	50.9	28.6
	699	Steenbock XV	2	109.1	54.9	30.4
	528	Pork I	1	122.5	54.6	32.4
	554	Pork I	2	118.1	49.3	28.9
		"Toxic" animals	—	151.0	48.6	14.1
Biotin	2.71	Steenbock XV	1	0.92	0.074	0.119
	2.69	Steenbock XV	2	0.92	0.058	0.101
	0.38	Pork I	1	0.67	0.068	0.057
	0.40	Pork I	2	0.70	0.055	0.053
		"Toxic" animals	—	0.87	0.065	0.032
Pantothenic acid	1088	Steenbock XV	1	89.2	6.4	37.7
	1047	Steenbock XV	2	70.2	6.0	32.1
	131	Pork I	1	45.4	6.5	37.8
	137	Pork I	2	39.7	5.8	30.9
		"Toxic" animals	—	69.4	6.9	16.3
Thiamine	145	Steenbock XV	1	10.5	1.2	2.4
	140	Steenbock XV	2	10.4	1.1	2.4
	130	Pork I	1	6.8	1.3	2.2
	136	Pork I	2	6.9	1.3	2.1
		"Toxic" animals	—	8.0	1.1	.

Only data on the occurrence of the vitamins in the maternal and fetal tissues of groups 3 and 4 will be presented at this time. These figures are given in table 4. Information on the concentration of the vitamins in tissues of younger animals will be reported in another paper.

Riboflavin

The normal reproductive performance of females receiving the Steenbock XV ration suggests that the daily intake of 90 μ g of riboflavin during pregnancy provides enough and possibly some surplus of this factor for normal reproduction. The fact that the respective concentrations of riboflavin in the fetal tissues and the maternal hepatic tissues agreed so closely at the termination of both the pregnancies is additional evidence that 90 μ g of riboflavin is ample for reproduction.

On the other hand, the 36 μ g of riboflavin provided daily by the Pork I ration did not allow deposition of riboflavin in hepatic or fetal tissues equal to that characteristic of animals receiving the stock ration. One might conclude, then, that the lower intake was not optimum during pregnancy. The difference in riboflavin intake provided by the two rations, however, seemed to have no influence on the concentration of this factor in the "carcass" tissues. This finding is in agreement with the work of others; the concentration of vitamins in the liver is a better indication of the state of nutrition of the animal than the vitamin content of other tissues.

The 4 females which developed toxemia of pregnancy contained approximately the same total quantity of riboflavin in their livers as the other animals receiving the Pork I diet, but due to the smaller weight of their livers the concentration of vitamin per gram of fresh tissue appears high. Since the young produced by such females were representative of fetal tissue on the 16th and 18th days of pregnancy and no young of toxic animals were available on the final day of pregnancy, no information was obtained on the effect of the toxic state of the female on the concentration of this vitamin in the fetal body.

While the low intake of riboflavin characteristic of animals receiving the Pork I ration may have had some bearing on the outcome of pregnancy, and particularly on the condition

of the young at birth, the degree of deficiency of this factor appears to be minor when compared with findings relating to biotin and pantothenic acid.

Niacin

No significant differences were found between the concentrations of niacin in the hepatic, "carcass", or fetal tissues of rats receiving the two types of rations, even though these rations provided quite different quantities of the vitamin. Regardless of the niacin intake of the mothers or the average number of young produced, the concentration of this factor per gram of fresh fetal tissue was strikingly constant, 28.6 to 32.4 μg per gram. This uniformity in the niacin content of the tissues was not surprising in view of the work of Ers-hoff ('46) on the dispensability of dietary niacin for reproduction. The present data seem to indicate that the niacin concentration of the tissues was normal in animals fed both rations, and that the disorders of reproduction observed in rats fed the Pork I ration were not related in any way to the niacin content of the diet or to the concentration of this factor in the various tissues. It was interesting to find that the embryonic tissues of toxic mothers on the 16th and 18th days of pregnancy contained very small amounts of niacin. It is regretted that no full-term young of toxic animals were available for comparison. Possibly the low concentration of this factor in the young of toxic females on the 16th and 18th days of pregnancy is typical of normal embryonic tissues at this age.

Biotin

The wide difference in the amount of biotin provided by the two rations, and the finding that the maternal stores of the animals fed the Pork I diet were low in this factor, lead one to suspect that a biotin deficiency was an influencing factor in the disturbances of pregnancy noted among animals receiving the Pork I ration. Since the young of such females contained only 50% as much biotin as did the litters of moth-

ers fed the Steenbock XV ration, it is likely that a deficiency of this vitamin also was important in affecting the survival of the young. This finding may explain the high mortality rate observed for the young of females fed this ration.

The limited intake of biotin provided by the Pork I ration may also prove of importance through its interrelationship with pantothenic acid, which also was low in this ration.

Pantothenic acid

The Steenbock XV diet was especially rich in pantothenic acid, providing over 1 mg of the vitamin daily as calcium pantothenate. Animals consuming this ration averaged approximately 89 μ g of pantothenic acid per gram of hepatic tissue at the end of the first pregnancy and their young contained over 37 μ g of the vitamin per gram. This high concentration of pantothenic acid noted in the young of control animals is in agreement with the findings of Henderson et al. ('42) and Unna and Richards ('42), who have suggested a high requirement for this vitamin in the young animal. When the rats were continued on the control diet for a second pregnancy, the concentration of the vitamin in the hepatic tissue diminished from 89 to 70 μ g per gram. At this time the average concentration of pantothenic acid in the fetal tissue was 32 μ g per gram.

In the case of the animals receiving the Pork I diet which supplied only 130 to 140 μ g of pantothenic acid per day, hepatic stores of the vitamin were markedly reduced, only 45 μ g per gram being present at the end of the first pregnancy. The concentration of this factor in the young, however, was comparable to that in the young of females fed the control ration. The maternal stores at this intake of pantothenic acid seemingly were being used in an effort to maintain high concentrations of the vitamin in the developing young. When the animals were remated for a second pregnancy, the liver stores of the vitamin were reduced still further (39.7 μ g per gram), although the average concentration of panto-

thenic acid in the 10 second litters was still close to the normal value of the fresh fetal tissues of the control rats.

In the group of 10 pork-fed animals that underwent second pregnancies, three females produced litters containing 10, 10, and 11 young. On the final day of pregnancy, when the animals were sacrificed, it was observed that these rats had gained only 67, 53, and 48 gm respectively, in contrast to increments ranging from 80 to 100 gm in the other 7 females. Pantothenic acid assays of the fetal tissues of the three animals revealed concentrations of 23.4, 23.9, and 23.8 μg of the vitamin per gram of fresh tissue. It is believed that these three females were sufficiently depleted in pantothenic acid to be unable to transfer ample amounts of it to their developing young, and that the fetuses were being resorbed at the time the mothers were sacrificed.

Since the concentration of pantothenic acid in fetal tissue was as high as 37 μg per gram in normal animals, it was surprising to observe that the embryonic tissues of females which became toxic on the 16th and 18th days of pregnancy contained only 16 μg per gram. The small amounts of fetal tissues present in the uteri of such animals, together with their low pantothenic acid concentration, suggest that a shortage of pantothenic acid was one of the causative factors in the resorptions that were occurring.

The importance of pantothenic acid in reproduction, together with the role of the vitamin and its interrelationship with biotin, are questions which are still unsettled. Nelson and Evans ('46) have reported that pantothenic acid is needed to prevent failure of implantation, resorptions, and birth of defective litters in rats. Emerson and Wurtz ('44) noted that a biotin deficiency was aggravated by superimposing a deficiency of pantothenic acid and, conversely, that the feeding of biotin appeared to lessen the severity of the syndrome associated with a lack of pantothenic acid. These findings have afforded some interesting speculations concerning the multiple deficiency encountered in pregnant ani-

to explain the toxic pregnancy and other abnormal symptoms noted. Wright and Welch ('44) have shown that the concentrations of pantothenic acid in the hepatic tissues of rats deficient in folic acid, biotin, and pantothenic acid did not return to normal after the administration of this vitamin unless biotin and folic acid were fed simultaneously. The findings of Pilgrim et al. ('42); Dorfman et al. ('42); Hills ('43); and Novelli and Lipman ('47) on the importance of pantothenic acid in the removal of pyruvic acid, together with those in the recent paper by Shive and Rogers ('47) relating to the function of biotin in the carboxylation of pyruvic acid to oxalacetic acid, may be the beginning of a better understanding of the role of these two factors in metabolism.

The development of the dramatic symptoms of toxemia encountered in 4 animals during these experiments is difficult to understand. Histological examinations of the tissues suggest that these changes may be brought about by some toxic substance produced in the resorbing fetal tissue (Armstrong and Swanson, '43). The fact that in almost every case in which females developed the pregnancy syndrome the animals were producing large numbers of young, coupled with the observation that the disorder appeared near the termination of pregnancy, may mean that the large fetal mass depleted of vitamin could not be completely resorbed without damage to the deficient mothers. A third possibility may be that shortages of biotin and pantothenic acid induce metabolic changes in the maternal rat of sufficient magnitude to cause death. On the other hand, pregnancy may merely increase the animal's need for these two factors. Also, the shortage of pantothenic acid may have damaged the adrenal gland sufficiently to produce the symptoms of toxemia directly. Histologic studies (Molsberry, '43) of the glands have shown that acute hemorrhagic necrosis accompanies the appearance of the toxic syndrome. These various possibilities are being studied.

Thiamine

Since the daily thiamine intake was much the same for all pregnant rats, it was surprising to find that the concentrations of thiamine in the tissues varied considerably between the groups of animals fed the two kinds of rations. The average vitamin concentration in the hepatic tissue of animals fed the Pork I ration was $6.8 \mu\text{g}$ per gram at parturition, approximately $\frac{2}{3}$ the concentration found in animals receiving the control diet. Since thiamine, biotin, and pantothenic acid are all recognized as functioning in carbohydrate metabolism, this lower concentration of thiamine in the livers of animals receiving the Pork I diet may mean that more thiamine was needed when the biotin and pantothenic acid intakes were low. Additional information regarding this point is being collected at this time. Note should be made of other experiments to be reported shortly which show that the glucose tolerance curves of pork-fed rats are abnormal. This abnormal response to glucose administration is intensified in toxic animals.⁹

SUMMARY

In an effort to determine the cause of unsatisfactory reproduction in rats fed a ration containing pork muscle and yeast as the main sources of protein and the B-vitamins, the concentrations of thiamine, riboflavin, niacin, biotin and pantothenic acid have been measured in the diets and in the tissues of pregnant animals. The occurrence of the 5 vitamins in hepatic, carcass, and fetal tissues on the final day of 1st and 2nd pregnancies has been determined in animals fed two types of rations. A modified Steenbock stock ration was used as the control diet, since it was known to promote normal reproduction.

Vitamin assays of the rations revealed that the animals which showed poor reproductive performance were consuming quantities of thiamine equal to those fed the modified stock diet, but that their riboflavin intake was somewhat

lower than that of animals fed the control ration. The greatest differences in vitamin intakes were observed in respect to biotin and pantothenic acid; the inadequate diet provided only 15% as much of these factors as was supplied by the stock diet.

The concentration of vitamins in the tissues indicated that a desirable intake of riboflavin during reproduction falls in the range of 36 to 90 μg per day.

Animals fed either ration contained large amounts of niacin in their tissues, and the concentration of this factor seemed independent of the dietary intake.

The concentration of biotin in both the maternal liver and the young of animals fed the ration providing minimal supplies of biotin was subnormal.

Animals receiving the inadequate ration were markedly depleted in pantothenic acid as judged by the low concentration of this factor in the hepatic tissue. They appeared to transfer large amounts of this vitamin to the developing young until severe maternal depletion occurred.

When dietary inadequacies of both biotin and pantothenic acid occurred, as seemed to be the case when the Pork I ration was given, the animals lost the ability to store thiamine in customary amounts in their livers, after dietary supplies had been transferred to the fetuses. Perhaps in a simultaneous deficiency of biotin and pantothenic acid a larger intake of thiamine is needed to permit deposition of normal quantities in the liver.

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REPRODUCTION AND LACTATION OF RATS RECEIVING PORK DIETS¹

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Purified rations have been found adequate for the complete life cycle of the rat, though a number of investigators have reported improvement in the lactation when various naturally occurring foodstuffs were added to the ration (Richardson and Hogan, '45; Vinson and Cerecedo, '44; Nelson and Evans, '47a; and Sporn, Ruegamer and Elvehjem, '47). Rations composed of corn, soybean oil meal, alfalfa leaf meal and minerals supplemented with the recognized nutrients have supported less satisfactory lactation than the purified rations (Spitzer and Phillips, '46). The requirements for the rat during reproduction and lactation have recently been reviewed (Schweigert, '47).

In studies conducted at Iowa State College for a number of years (Swanson, Armstrong and Nelson, '43; and Swanson and Nelson, '40) another type of diet containing natural products which would be expected to be adequate failed to support rats through the entire life cycle. Rations containing autoclaved dried pork were markedly inferior for growth, reproduction and lactation to similar rations containing beef. The females receiving pork diets showed toxemia of pregnancy with frequent deaths, fatty livers, gastric ulcers, and

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complete reproductive failure in the 2nd generation. In more recent work (Swanson, '44) synthetic vitamins have replaced 5% yeast which was used in the earlier studies as the source of B vitamins. Under this diet mothers gave birth more frequently to normal young but in most cases lactation failed. Since addition of fresh liver to these rations permitted the rats to reproduce and lactate normally, it was concluded that the pork diets were deficient in some nutrient or nutrients required for these functions.

Since 1944 work has been in progress in our laboratories in an effort to compare the value of pork with beef and other sources of protein in supporting rats during the reproductive cycle. Except for a few cases, these studies have included only parental generation rats with two litters for each female. The findings, though variable and difficult to interpret, indicate that cooked pork is sometimes inferior to casein- or beef-containing rations, particularly for lactation. Frequently the performance of rats receiving pork diets is quite comparable to that of animals receiving rations with other sources of protein. Little or no difficulty has been encountered with toxemia of pregnancy or non-viable young.

EXPERIMENTAL

Care of animals

Twenty-one-day-old, weanling, female rats obtained from Sprague-Dawley were used in all studies. In the first 4 experiments 6 rats per group were used, but in experiments V and VI each group contained 8 animals. Three or 4 rats were kept in each raised screen-bottomed cage. They received the experimental rations ad libitum, until removal to individual cages 5-7 days prior to parturition. Weights were recorded once each week until after mating and at least every three days during the gestation period.

In the first three experiments the females were mated with stock-colony males at 80 days of age. Mating was delayed until 100 days of age in the later experiments. Pregnant

females were placed in individual cages with a sheet metal floor and wood shavings for a nest. The young were counted, but not handled until the day following birth, when the mother and young were weighed and the number of young reduced to 6 per litter. Weights of young and mothers were recorded at weekly intervals, i.e., on the 8th, 15th, and 22nd days following parturition. The young were weaned on the 22nd day. The females were continued on the same ration in most cases and mated again one month later.

Composition of rations

Minor changes in the methods of compounding, handling and storing rations were made as the studies progressed. These changes are described in the paragraphs which follow. All rations were compounded isocalorically to maintain a constant protein content and the same mineral and vitamin composition, exclusive of these elements furnished by the meats.

The basal ration had the following composition: Meat or casein to provide 15, 24 or 30 gm of protein, salts IV (Phillips and Hart, '35) 4 gm, corn oil 5 gm, sucrose to make approximately 410 calories, thiamine hydrochloride 0.25 mg, riboflavin 0.30 mg, pyridoxine 0.25 mg, nicotinic acid 1.5 mg, DL-calcium pantothenate 2 mg, choline chloride 100 mg, inositol 100 mg, para-aminobenzoic acid 30 mg, biotin 0.01 mg, pteroylglutamic acid 0.02 mg, and α -tocopherol 10 mg. In addition, two drops weekly of a mixture containing corn oil, halibut liver oil, α -tocopherol and menadione were given. The daily intakes of fat soluble vitamins from this source were approximately 400 I.U. of vitamin A, 4 I.U. of vitamin D, 0.5 mg of α -tocopherol and 0.04 mg of menadione.

In the first two experiments the water soluble vitamins were added to the ration in solution at levels equivalent to those shown above, except that biotin was added at 0.002 mg per 100 gm and no pteroylglutamic acid was used. The nicotinic acid was increased from 0.25 mg to 1.5 mg per 100 gm at the beginning of experiment V. The additional α -tocopherol ad-

ministered by dropper was begun with the 2nd litter of experiment III and continued throughout subsequent experiments. The vitamin levels were varied in experiment VI; group 1 in this series received the above vitamins at twice the concentrations shown above.

Preparation of meats

Meats, procured from a local packing house soon after slaughter, were roasted or otherwise processed as indicated below. Skinned, boned hams weighing approximately 7 pounds were roasted in an electric roaster at 350°F. until the meat reached an internal temperature of 180°F. This required approximately 4 hours of cooking. After trimming off the excess fat, the pork was finely ground and stored at -5° until used. Cuts of beef round were roasted in a similar manner, ground and stored. Analyses of a number of cuts of meat thus cooked indicated an average composition of: protein 33%, fat 8%, moisture 57% for beef; and protein 27%, fat 22% and moisture 48% for pork. When fresh, dried or extracted meats were fed, protein (by Kjeldahl nitrogen) fat and moisture were determined on each such preparation until such time as reasonably accurate average figures were obtained.

In experiments I and II the meats were prepared in large quantities, ground, stored for several weeks at -5°, and incorporated into the rations as needed. In all other studies the pork was procured weekly and the beef weekly or bi-weekly and cooked after one to three days of cold storage. Cooked meat not fed within 7-10 days after cooking was discarded. All rations were made fresh at intervals of 4-10 days and all were stored at a temperature of -5° to +5°.

The results of experiment II suggested that changes resulting from rancidity of the fat might be contributing to the poor results with pork rations. The effect of storage time on the oxidative rancidity was checked by determining the peroxide numbers of a number of meat samples and of the meat-containing rations stored for various periods of time. The

results of these determinations (table 1) indicated that roast pork, ground and stored, developed small amounts of peroxides in 6 days. In 12 days considerable oxidative rancidity had occurred. Likewise, the ether extract of roast pork showed a marked increase in peroxide content after storage. To insure that this factor would not complicate later experiments, meats were procured and cooked frequently enough to preclude the possibility of undue oxidative rancidity.

The solvent extractions required for the preparation of rations for experiments III and VI were made at monthly intervals, using meats (raw or cooked) which had been ground

TABLE 1
Peroxide numbers for meats and meat-containing rations stored at -5°

SAMPLE	PERIOD STORED	AVERAGE PEROXIDE NUMBER
	<i>days</i>	<i>per kg of fat</i>
Roast pork	1	4.1
Roast pork	6	3.5
Roast pork	12	35.5
Roast pork	30	47.5
Pork fat (ether extract of roast pork)	120	53
Fresh ground ham	105	6.5
Roast pork ration	5	4.2
Roast pork ration	30	22.6
Beef and pork fat ration	30	28

and dried in a thin layer in a current of air at 50° . The dried meats were covered with Skelly-solve B ($50-70^{\circ}$) or ethyl ether and stirred for a few hours. The solids were allowed to settle and the extract decanted off. This extraction of the residue was repeated twice and the three extracts combined and concentrated *in vacuo*. Removal of the last trace of solvent was accomplished by bubbling nitrogen through the melted fat until there was no detectable odor of the solvent present. The crude fats so prepared were stored in a stoppered brown bottle in the dark at -5° until mixed with the rations. The residues were dried at 50° for a few hours, ground to a fine powder and stored at -5° in a closed con-

tainer until used. For experiment II a continuous extractor was used for the preparation of the ethyl ether extract.

RESULTS

The data on the reproduction and lactation of rats in all experiments are presented in table 2. In the 1st experiment the rations were patterned after those of Swanson et al. ('43) in that two levels of protein were provided, 15 and 30%. All meats were cooked, ground and stored until needed, sometimes for several weeks. The pork-containing rations failed to support good reproduction and lactation. A large number of the young were born dead or died soon after birth; they were small and grew poorly, especially when the mothers received the low level of protein. Impaired lactation, particularly in the 2nd litter, was evident in the groups receiving pork rations. A large number of the females in all groups contracted upper respiratory infections and died or failed to mate the 2nd time. One female receiving the 30% pork protein ration died during parturition. Except for this one case, no manifest difficulties arose at parturition and the severe symptoms reported by Swanson and Nelson ('40) were not noted.

In the 2nd experiment, one level of protein (30%) was used. The ether extract of cooked pork was added to the beef-containing ration (group 5). Group 6 differed from group 1 only in that the hams were roasted fresh each week. As in experiment I, respiratory infection was a complicating factor, reducing the number of females to one or two in some groups for the 2nd mating. For this reason, data are not presented for the 2nd litter in these two experiments.

In all subsequent experiments the incidence of respiratory infection was lower. Although minor colds, which usually cured spontaneously, occurred following the 1st litter, it was felt that they did not complicate the results. Attempts to correlate the reproduction and lactation performance with the occurrence of these infections were not successful.

For example, in experiment V, 2nd litter, approximately 33% of the beef-fed rats had pulmonary congestion as shown by autopsy immediately after losing or weaning young, while 41% of the pork-fed rats and 12% of the casein-control animals were infected. In all experiments the incidence of lung infection was approximately 30%, but rarely was it severe enough to result in weight loss by the females.

In experiment II all rations contained 24% protein, and this level of protein was adopted for all subsequent experiments. Unused rations and cooked meat were discarded one week following cooking to insure that rancidity or spoilage would not result. All uneaten rations were removed from the feed cups daily and the cups were carefully washed twice weekly. Seldom was any evidence of spoilage detected in the uneaten portions. Changes in the rations of some groups were made immediately after the 1st litter was weaned. The 4% liver extract, which had no pronounced effect on the reproductive performance of rats receiving roast pork as the source of protein in the 1st litter (group 4), was replaced by 2 ml of fresh milk per female rat per day for the 2nd litter (group 4a). The petroleum ether residue of roast pork (group 5) was replaced by the ethyl ether residue (group 5a), and the petroleum ether extract of roast pork (group 9) was replaced by the ethyl ether extract (group 9a). The level of the "B" vitamin mixture was doubled for the 2nd litter for the groups receiving the high lard ration (groups 10 and 10a).

In all groups the performance of females was better than in the two previous experiments. Nearly all became pregnant. In the 2nd mating, vaginal smears were made daily from the time of mating until pregnancy was established. Only one resorption was recorded. Change in weight appears to be an adequate indication of pregnancies and resorptions in such studies. The control group, receiving a 24% casein ration, successfully reared a major portion of the young born, giving results quite comparable to those obtained by other workers in this laboratory using animals from the same source (Sporn et al., '47). Furthermore, most of the females receiving ra-

TABLE 2
Summary of reproduction experiments

EXP. GROUP NO.	RATION COMPOSITION	REPRODUCTION: % FEMALES HAVING YOUNG			VARIABILITY: % OF YOUNG ALIVE AT 24 HOURS			LACTATION: % OF YOUNG WHICH WERE WEANED			FRACTION OF YOUNG WEANED 1-6			WEIGHT OF YOUNG AT 1 DAY		WEIGHT OF YOUNG AT WEANING
		1st litter	2nd litter	Ave.	1st litter	2nd litter	Ave.	1st litter	2nd litter	Ave.	1st litter	2nd litter	Ave.	1st litter	Ave. both litters	
I	1 Roast pork, 15% protein	100	80	90	80	20	50	25	0	12	1/6	0/4	gm			
	2 Roast beef, 15% protein	83	43	62	70	80	75	73	100	87	3/5	1/2	5.3	5.9	54	
	3 Roast pork, 15% protein + corn meal	100	100	100	90	41	65	63	55	54	2/5	1/5	5.7		38	
	4 Roast pork, 30% protein	83	20	52	75	25	50	48	0	24	2/5	0/1	5.6	4.1	41	
	5 Roast beef, 30% protein	100	40	70	95	80	87	88	100	94	5/6	2/2	5.7	4.2	42	
	6 Casein control, 24% protein	100	100	100	97	98	98	81	83	82	5/6	4/5	6.0		37	
II	1 Roast pork	60	20	40	92	100	96	71	100	86	2/3	1/1	5.3		31	
	2 Roast beef	80	25	52	42	20	31	60	1/4	1/1	5.5	34	..	
	3 Roast pork + corn meal	0	0	0	0	0	0	
	4 Ether res. roast pork (dried)	100	100	100	91	97	94	79	96	87	4/5	4/5	5.7		36	
	5 Roast beef + pork fat (ether ext.)	50	20	35	45	67	56	44	1/3	0/1	5.1	37	..	
	6 Fresh roast pork	100	25	63	83	36	60	49	3/8	0	5.1	39	..	
III	1 Frozen pork (uncooked)	85.7	80	83	63	53	58	58	100	79	2/6	2/4	5.9		48	
	2 Dried pork (uncooked)	66.7	100	83	67	43	55	89	55	72	3/4	2/6	5.8		47	
	3 Roast pork	66.7	100	83	97	73	85	92	68	80	4/4	3/5	5.8		45	
	4 Roast pork + 49% 1:20 LE ^a	100	..	100	83	..	83	64	..	64	3/6	..	5.6		47	
	4a Roast pork + 10 ml milk/day	..	100	100	..	72	72	44	44	44	..	2/6	5.2		44	
	5 Pet. ether res. roast pork	83	..	83	67	..	67	84	..	84	3/5	..	5.9		40	
	5a Pet. ether res. roast pork	..	100	100	..	37	37	..	0	0	..	0/4	4.5		..	
	6 Pet. ether res. dried pork (uncooked)	100	100	100	81	41	61	75	83	79	3/5	2/4	5.9		41	
	7 Casein control	100	100	100	76	68	72	74	73	74	4/6	4/6	5.7		36	
	8 Casein + pet. ether ext. dried pork	100	100	100	79	58	68	79	54	67	4/6	2/6	5.7		39	
IV	9 Casein + pet. ether ext. cooked pork	100	..	100	79	..	79	34	..	34	2/6	..	5.6		41	
	9a Casein + ether ext. cooked pork	..	83	83	..	88	88	..	62	62	..	3/5	5.4		41	
	10 Casein + 23% lard	67	..	67	46	..	46	8	..	8	0/4	..	5.4		18	
	10a Casein + 23% lard doubled vit.	..	100	100	..	61	61	67	67	67	..	2/5	6.3		42	
	1 24% casein, 5% corn oil	100	100	100	93	100	96	74	92	83	4/6	6/6	6.1		41	
All doubled vit.	2 24% casein, 5% corn oil + 23% non stab. lard	100	100	100	76	88	82	84	88	86	5/6	3/5	6.5		47	
	3 24% casein, 5% corn oil + 23% stab. lard	100	100	100	79	86	82	86	42	64	5/7	3/7	6.0		46	

V	1	Roast beef	100	100	100	93	97	95	92	85	89	7/8	7/8	6.1	37
	2	Roast beef doubled B-vit.	100	100	100	63	82	73	56	71	64	5/8	6/8	6.3	43
	3	Roast beef doubled B-vit. + 0.5 gm liver/day	100	100	100	83	90	87	75	95	85	6/8	7/8	6.1	40
	4	Roast pork	100	100	100	78	72	75	30	22	26	3/7	1/6	5.9	42
	5	Roast pork doubled B-vit.	100	100	100	68	82	75	30	30	30	2/8	1/6	5.7	40
	6	Roast pork doubled B-vit. + 0.5 gm liver	100	100	100	93	76	84	55	70	63	4/7	4/7	6.2	41
	7	Casein + 5% corn oil	100	100	100	61	90	76	15	62	38	1/8	3/7	5.6	40
	8	Casein doubled B-vit.	100	100	100	89	90	90	50	63	57	3/8	5/8	5.8	33
	9	Casein doubled B-vit. + 5 gm liver	100	100	100	86	94	90	67	67	67	5/7	5/7	5.9	33
VI	1	Roast pork doubled B-vit.	88	100	94	70	76	73	85	92	89	4/7	5/6	6.9	59
	2	Roast pork with levels of 6 B-vit. adjusted. No added 4 B-vit. ⁴	75	80	78	62	76	69	83	88	86	4/6	4/4	6.3	50
	3	Roast pork with levels of 6 B-vit. adjusted and 4 B-vit. at regular levels	100	100	100	86	84	85	97	95	96	7/8	7/8	6.9	57
	4	Pet. ether residue of roast pork with adjusted B-vit. as in grp. 3	88	100	94	92	89	91	93	80	87	7/7	6/7	6.6	46
	5	Roast pork + 5% alcohol extracted casein ⁵ (vit. as in grp. 3)	88	100	94	84	72	78	100	100	100	6/7	5/8	6.9	54
	6	Roast pork + 10% fresh beef fiber (vit. as in grp. 3)	88	84	86	66	95	81	100	70	85	4/7	4/6	6.8	55
	7	Roast pork + 1% liver ext. ⁶ (vit. as in grp. 3)	88	100	94	90	64	77	100	100	100	6/7	4/6	7.0	57
	8	Roast pork (15% protein) + roast beef (12% protein)	88	75	82	93	100	97	100	83	92	7/7	6/6	6.4	55
	9	Casein + 6 B-vit. doubled + 4 B-vit. single level	88	100	94	95	84	90	95	80	88	7/7	6/8	6.9	44

¹ Denominator of fraction is the number of females which gave birth to young.

² Figures for one litter only for experiments I and II.

³ Wilson's 1:20 Liver Powder.

⁴ See text.

⁵ SMAO purified casein was extracted 10 times by refluxing 24 hours with 3-4 volumes of 95% ethanol each time.

⁶ Sharpe and Dohme product no. 2505.

tions containing pork or preparations of pork did almost as well. From 80 to 100% of the mated females gave birth to young, and 55 to 80% of the young survived 24 hours. The lactation was fair, one-half to two-thirds of the females weaning 4-6 young. Exceptions were the animals receiving the ethyl ether residue of cooked pork, the petroleum ether extract of the cooked meat, and those receiving the milk supplement. In these cases, one-third or less of the females weaned 4-6 young. Doubling of the vitamin supplement added to the high lard ration caused marked improvement, especially in lactation. Whether the requirements of some vitamins for lactation are higher than the amounts fed when the ration contained much fat, or there was enhanced destruction of one or more vitamins during the one-week storage of them already mixed in the ration, has not been determined. That one of the two was involved is indicated by the differences in the results obtained with groups 7 and 10.

In the next experiment (IV), started while experiment III was still in progress, high lard diets were fed in an effort to determine if the high fat level was resulting in oxidative changes. Five per cent corn oil rations were compared with high levels of stabilized and non-stabilized lard.² The lard replaced approximately 60% of the sucrose isocalorically to give a ration containing 30% fat by weight (23% lard). The B-vitamins were doubled in all groups. In this experiment, and in the two which followed, the 1st mating was delayed until the females reached 100 days of age. All of the females became pregnant, most of them produced normal, healthy young, and most of the young survived for 24 hours. The lactation index was only 42% for the 2nd litter in the group receiving stabilized lard. The reason for this inferiority was not evident. Three of the females lost all of their young in the 1st week and none had a respiratory infection. The performance of the casein-corn oil control group was better than in the previous experiment, possibly because of the more

² Kindly supplied by Armour and Company, Chicago, Illinois.

advanced age at the time of mating.³ The results indicated that when fat is substituted isocalorically for carbohydrate it does not markedly affect the reproductive functions of rats receiving 24% casein rations in the presence of adequate levels of vitamins.

Because experiment III (groups 10 and 10a) indicated increased destruction of, or increased requirement for one or more B-vitamins in rations high in lard, the next experiment was planned to ascertain the effect of supplementing casein, beef and pork rations with addition of B-vitamins and fresh liver (see table 2). It will be noted that in contrast to the results of experiment III, animals receiving pork rations failed to lactate properly in both litters. Doubling the B-vitamins had no effect, while 0.5 gm of fresh beef liver per day resulted in some improvement in this function. Inferior lactation in the 1st litter by females receiving the casein control ration was improved by doubling the vitamins, and was further improved by supplementing with fresh liver. Increased vitamin levels appeared to increase the mortality of young and to decrease the lactation of the females receiving the roast beef.

The observation that increasing B-vitamin levels had a detrimental effect on the beef-fed rats but improved the casein and pork-fed rats, and the findings of Richards ('45), indicate the possibility of an imbalance of vitamins being responsible for the inferiority of pork to beef and casein in certain experiments. This possibility was investigated in experiment VI.

In the previous experiments all of the vitamins were added without correcting for those provided by the meats in the rations. In this experiment, B-vitamins were added to the rations, as indicated in table 2, in three different patterns: (1) At twice the regular levels in addition to those provided by the meats; (2) only thiamine, riboflavin, nicotinic acid,

³ Rats from this source receiving stock diets have been found to mate and reproduce more successfully if they are at least 100 days of age (R. K. Meyer, unpublished).

pyridoxine, calcium pantothenate and choline at twice the regular levels, taking into account the amounts of these 6 vitamins present in the meats; and (3) the same as (2) except that regular levels of biotin, folic acid, inositol and *p*-aminobenzoic acid were provided in addition to those furnished by the meats. Other variations included the replacement of a portion of the pork by casein and beef, and supplementation of the pork rations with fresh liver or a liver extract. In contrast to experiment V, lactation was good in all groups (85% or more). In some groups one or two females failed to become pregnant and in a few instances the young were less viable. Unusually good performances in all respects were obtained with casein, a pork-beef mixture or petroleum ether-extracted pork as the protein sources. The improved performance was also reflected in larger young at birth and at weaning than had been obtained previously (table 2). The young from mothers on lower fat rations (groups 4 and 9) were smaller than those in other groups. There is some indication that the young survived the first two days of life better when the mothers were smaller, but this correlation does not exist in all groups. The uniformly good results in this experiment prevented the observation of any possible influences of the vitamin imbalance which may exist in these rations.

From the more or less uniform results obtained with casein and beef in the last 4 experiments, and the extremely variable results with pork, it seems probable that the quality of the pork was an important factor in these studies. In experiment III the roast pork appeared to maintain nearly an adequate nutritional state for the reproductive cycle; in experiment V, however, roast pork failed to support good lactation unless supplemented with fresh beef liver. Again, in experiment VI roast pork supplied the necessary nutrients for a relatively good reproductive performance. It should be pointed out that group 1, experiment VI, and group 5, experiment V, received identical rations except that different batches of meat were used. The cuts of meat used in all experiments except I and II were obtained from market hogs and were purchased often

enough to represent a cross section of the hogs sold in this locality at the time.

The growth rates of the females receiving pork rations were never inferior to groups in the same experiment receiving other rations. Often they grew slightly better than the control groups receiving casein as the protein. The average weekly growth rate was approximately 25 gm (20–28 gm) for all experiments during the first 6 weeks. The average number of young born per female was also not significantly affected by the rations in these studies. The averages in most groups were 7–9 young per litter, the 2nd litter containing the same number or slightly fewer than the 1st.

DISCUSSION

Why pork should be inferior to beef in the nutrition of the rat is not evident. The most notable difference in gross composition, the lipid content, does not appear to be the explanation, since lard at a fat level comparable to that provided by pork was not detrimental when casein was the protein. Confirming the findings of Swanson and Nelson ('40), rancidity of fat present in the pork rations is not responsible for this inferiority. Deuel et al. ('47) report better reproduction and lactation with some fat in the ration. Increasing the level above 5% caused no improvement, but resulted in slightly higher mortality during the nursing period. Anthony ('44) reported satisfactory reproduction and lactation on a low fat diet. When rice bran concentrate was used as the source of water soluble vitamins and 1% linoleic acid as the sole lipid source, satisfactory growth, reproduction and lactation were obtained. When rice bran concentrate was replaced by the crystalline vitamins, satisfactory reproduction was found with 1–2% ethyl linolate, but growth and lactation were improved by addition of 30% butterfat or cottonseed oil. Nelson and Evans ('47b) reported a deleterious effect on lactation when the fat content of the ration was increased. They did not make their substitutions of fat for carbohydrate on an isocaloric basis, however, and the influence of increased fat

levels on lactation may have been the result of the effective lowering of protein, salts or vitamins. These ingredients were reduced to approximately $\frac{2}{3}$ of the level fed on the high carbohydrate and high protein diets when calculated on an isocaloric basis.

The protein quantity and quality do not appear to be responsible for the difference between pork and beef. Swanson and Nelson ('40) found no significant change in the biological value of pork protein during cooking. Addition of liver, which did not alter the biological value, improved the nutritive value markedly as judged by reproduction and lactation performances. Pork and beef protein appear to be very similar in amino acid composition and in biological value (Block and Mitchell, '47).

The variation from one experiment to another and the improvement in lactation when liver was added to the pork rations indicate a dietary inadequacy or imbalance. The improvement of lactation on casein rations by doubling the B-vitamins (groups 7 and 8, experiment V) and failure to get improvement by this means with the pork rations suggest again an imbalance of vitamins or the presence of a substance or substances in casein and beef not present in pork muscle.

Attempts to use the short-term method (Nelson and Evans, '47b) of determining the effects of supplements on lactation were not successful in these studies. Weight increases of the mothers shifted from stock diets to the meat-containing rations at parturition were considerable in all cases. The variable results with pork may be related to the fact that hogs are monogastric animals and the pork muscle may not be supplied with ample quantities of unknown factors produced by the microorganisms in the rumen of ruminants.

It should be pointed out that these results cannot be applied to human nutrition, since no one would receive all of his protein from pork, nor would he consume pork at every meal. The experiments reported here are abnormally balanced in order to produce conditions which will allow demonstration of the differences between pork and other materials as sources

of protein. The variable factor may be the same as that encountered in several other studies, with various types of non-purified rations.

SUMMARY

Female rats fed rations containing pork as the source of protein mated normally and gave birth to apparently normal young, but in some cases failed to rear them. The primary defect appeared to be in lactation. Whereas casein-fed rats usually reared an average of approximately $\frac{2}{3}$ of the young and beef-fed rats 80-90%, pork-fed animals reared less than $\frac{1}{3}$ of their young in certain experiments, indicating poor lactation. In other experiments the lactation was comparable to that obtained with rations containing beef.

When lactation failures were encountered with pork rations, increasing the B-vitamins had no effect, but fresh liver restored the lactation to that obtained when beef or casein was fed.

The levels of B-vitamins commonly used in growth experiments were found inadequate for optimum reproduction and lactation performance with casein as the source of protein, especially when the fat level was high.

Attempts to obtain a defatted pork preparation which would approach the beef or casein rations in supporting lactation were not successful.

ACKNOWLEDGMENTS

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THE CHOLESTROL CONTENT OF COWS' MILK¹

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During the past few years interest in cholesterol as it relates to human physiology has markedly increased. One of the primary reasons for this is the suggested association between the development of blood vascular disorders and the cholesterol intake. A number of references in the popular press have indicated a relation between the intake of cholesterol and some diseases of the circulatory system (Leary, '44; Pasquarelli, '46; Dock, '47). Although many papers on this subject have appeared since Bischoff ('32) made a thorough evaluation of the available literature, his conclusion that there is very little valid evidence of the existence of any such relation still appears tenable.

In addition, cholesterol is being prominently mentioned as a precursor of various steroid hormones (Bloch, '45; Perlman and Leonard, '47), while the ester form has been suggested as the blood precursor of the fat in milk (Saarinen, '44). A great deal of work is being done on the relation of the cholesterol intake to the development of fatty livers (Loizides, '38; Best and Ridout, '36).

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The increasing interest being shown in cholesterol prompted a critical evaluation of the amount of this substance in milk. Although there are a number of reports on the level of cholesterol in cows' milk, there is no indication as to its variation in the milk from different cows or in that from the same cow on different occasions. The work reported here was undertaken to supply this information.

ANALYTICAL METHODS

The Schoenheimer and Sperry ('34) method for the analysis of free and ester cholesterol in blood was adapted for this work. In later reports, the Columbia group (Sperry, '38; Sperry and Brand, '43) suggested that the acetic anhydride and sulfuric acid be mixed and then kept cold in an ice bath until used. After a number of trials, however, it was decided to follow the older method. According to this procedure, the sulfuric acid was slowly added to the colorimeter tube containing the acetic anhydride and the digitonin precipitate which was dissolved in acetic acid. The tube was vigorously shaken while the sulfuric acid was added and the shaking continued for about a minute before the tube was placed in a water bath maintained at 25°. All readings were made with an Evelyn photoelectric colorimeter at intervals between the 27th and 37th minute after the addition of the sulfuric acid to the tube. In each case, the lowest galvanometer reading secured during this interval was used; it corresponded to the maximum color development. The cholesterol analyses were not started until experiments indicated that from 90 to 105% of the cholesterol added to milk could be recovered. All analyses were made in duplicate.

The butterfat in the milk was determined by the routine Babcock test. These analyses were made on the Farm Campus. The statistical evaluation of the data involved the analysis of variance as described by Snedecor ('46) ².

² We are grateful to Dr. W. O. Caster for aid in the statistical analysis.

EXPERIMENTAL ANIMALS

The cows used in this study were a part of the regular University Farm herd. Most of them had freshened within the three-month period preceding the first milk sample, while two of them were up to a month further along in their lactation period (table 1). In the early spring, when this study was initiated, the cows were kept in the barn. The milk samples obtained at that time are labeled "winter milk." During the summer, the animals were kept on pasture supplemented with concentrates. The samples obtained

TABLE 1

*Description of cows. Free and total cholesterol and butterfat in milk.
(The milk samples were collected on March 24)*

COW	AGE	LAST CALVING	CHOLESTEROL		FAT
			Free	Total	
	<i>years</i>	<i>date</i>	<i>mg/100 ml</i>		<i>%</i>
Holstein					
474	9	2/20/47	9.8	9.9	2.4
480	9	2/ 3/47	9.5	9.5	3.3
838	3	1/15/47
Jersey					
297	4	1/ 6/47	14.1	14.1	4.9
275	6	12/26/46	15.0	15.0	5.0
293	4	12/10/46	11.5	11.6	3.9
Guernsey					
639	6	1/28/47	10.5	10.4	3.0
672	2	2/26/47	10.4	10.3	3.7
658	4	11/19/46	.	.	.

at that time are called "summer milk." Six separate samples were analyzed at various intervals in each of these periods.

While the cows were kept indoors, they received alfalfa hay ad libitum, corn silage, and a grain mixture composed of corn, oats, barley, linseed oil meal, bone meal and salts. The cows were put out on pasture May 16, 1947, at which time the winter ration was reduced by one-half.

The cows were milked twice each day. On each occasion, the milk used in this work was obtained in the morning and was a true composite of that entire milking.

TABLE 2
*Cholesterol and butterfat in cow's milk*¹

Cow no.	HOLSTEIN						JERSEY						GUERNSEY					
	474		480		558		297		275		293		639		672		658 ²	
	Ch.	Fat	Ch.	Fat	Ch.	Fat	Ch.	Fat	Ch.	Fat	Ch.	Fat	Ch.	Fat	Ch.	Fat	Ch.	Fat
	Winter milk																	
3-24	9.8	2.4	9.5	3.3	7.4	2.4	14.1	4.9	15.0	5.0	11.5	3.9	10.5	3.0	10.4	3.7	12.7	4.7
4-17	9.6	2.5	9.3	2.9	7.8	3.2	12.1	3.8	14.2	5.0	9.9	1.9 ³	12.9	3.6	9.1	3.0	12.2	3.0
4-30	8.5	2.0 ³	8.4	2.0 ²	7.4	1.6 ³	12.8	4.7	12.4	4.3	16.8	5.8	13.8	4.4	9.3	1.8 ³	12.8	3.8
5-12	9.0	2.8	8.8	2.8	8.2	3.5	13.0	4.8	15.2	5.5	11.6	1.8 ³	14.7	4.2	9.5	3.8	12.2	4.4
5-21	8.2	3.1	11.1	4.7	7.0	3.3	11.5	5.2	13.3	5.6	15.6	4.9	16.0	4.8	12.8	4.8	11.8	4.8
5-30	9.0	2.5	9.5	2.4	8.7	2.1 ³	14.3	4.2	15.0	5.5	13.1	4.4	11.8	3.4	12.0	3.3	12.7	5.6
	Summer milk																	
7-9	10.1	2.7	8.8	2.5	8.4	3.3	12.6	4.8	11.2	5.7	13.5	4.7	8.0	4.5	9.1	3.6		
7-16	9.8	2.4	10.1	2.3	6.9	3.1	10.6	4.5	13.2	5.7	13.8	5.6	8.7	4.2	9.2	3.4		
7-23	8.3	2.6	11.7	3.0	6.7	3.2	9.3	4.7	13.2	5.2	11.6	5.5	10.3	4.4	11.7	4.0		
8-5	9.7	2.9	15.3	3.2	8.6	2.8	12.4	4.6	13.3	5.2	14.4	4.9	13.9	4.5	11.0	3.7		
8-12	16.8	3.8	16.6	3.4	8.5	3.1	11.6	6.2	13.3	4.6	12.4	4.4	10.1 ¹	4.2	11.3	4.6		
8-18	8.8	2.2	14.2	4.0	9.0	3.0	11.5	3.4	12.4	3.6	14.0	5.0	13.6	4.5	11.8	3.8		

¹ The fat is expressed as grams per 100 ml of milk. The cholesterol is expressed as milligrams of total cholesterol per 100 ml of milk.

² Cow 658 was sold before the experiment was completed.

³ The values followed by ³ are explained in the text.

RESULTS

The results of the analyses of the milk for both the free and the total cholesterol are given in table 1. The agreement between the two sets of values was so good that there was no reason to believe that any ester cholesterol was present in the milk. For this reason, the subsequent analyses for cholesterol involved only the precipitation with digitonin without any preliminary saponification.

The values for the cholesterol and butterfat in the milk samples are given in table 2. The results of the statistical

TABLE 3¹
Variations in the cholesterol content of milk

MILK	ALL COWS	HOLSTEIN	JERSEY	GUERNSEY ²
Within-cow variation				
Winter	11.4 \pm 1.50	8.7 \pm 0.80	13.4 \pm 1.98	11.9 \pm 1.62 12.4 \pm 1.50
Summer	11.3 \pm 1.56	10.5 \pm 2.05	12.5 \pm 1.01	10.7 \pm 1.28
Inter-cow variation				
Winter		8.7 \pm 1.14	13.4 \pm 1.93	11.9 \pm 2.45 12.4 \pm 1.97
Summer		10.5 \pm 3.03	12.5 \pm 1.39	10.7 \pm 1.17

¹ The mean values together with their deviations expressed as mg/100 ml of milk are given in each case.

² The first of the 2 values after Guernsey is the mean of 2 cows; the second is the mean of 3.

analysis of these data are presented in tables 3 and 4. From these it can be seen that the mean cholesterol level in the milk was 11.4 mg per 100 ml for the winter samples and 11.3 mg for the summer milk. When the values for all the cows are thus considered, there is no significant difference between the summer and the winter milk. As will be brought out later, there are some slight breed differences for the two seasons.

There is a slight day-to-day variation (variation between days, table 4) in the cholesterol content of the milk during

the summer; this variation is still less in the winter. The differences in the cholesterol level in the milk from the individual cows within the same breed are significant. This is particularly true for the summer, when the F test shows a significance beyond the 1% level.

The milk from the different breeds showed a significant difference ($P < 0.01$) in levels of cholesterol during the winter. In contrast to this, the summer milk shows no significant breed differences. This is due to the fact that the day-to-day variation was barely significant in the summer, while the differences in the cholesterol levels for the milk from cows within the same breed were so great that the

TABLE 4
Statistical evaluation of results¹

SOURCE OF VARIATION	WINTER		SUMMER	
	F test	Significance	F test	Significance
Variation between days	0.59	No significance	2.58	*
Variation between cows	2.84	*	6.62	**
Variation between breeds	16.23	**	1.26	No significance

¹ The significance of the F test is given according to the following notation: * = between the 5 and 1% level of significance, ** = beyond the 1% level of significance.

breed differences were completely overshadowed. This is perhaps more understandable when it is realized that the mean cholesterol levels for the winter milk from the various breeds ranged from 8.7 to 13.4 mg per 100 ml, whereas in summer the means ranged only from 10.5 to 12.5 mg (table 3). In the latter period, the deviation for any mean was usually greater than the difference between the respective means. For this reason, the breed differences in the summer were insignificant.

The seasonal variation in the cholesterol content of milk was not the same for the different breeds. For the Holstein milk, the difference in the cholesterol level for the two seasons was barely significant as shown by an F test of 25

(table 4). The difference for the Jersey milk was also only slightly significant. A similar analysis for the Guernsey milk was not made since one of these cows was sold in the middle of the experiment. The biological significance of these differences is open to question, since the milk from the Holsteins showed a lower mean value in winter than in summer and the Jersey milk exhibited just the reverse. These differences for the two breeds were of the same order of magnitude, since the mean cholesterol levels for all cows regardless of breed were the same for both seasons.

There was a considerable degree of correlation between the butterfat and the cholesterol contents. The correlation coefficient for this relationship in the milk from all cows throughout the entire experiment was 0.64, which was highly significant far beyond the 1% level. For the winter milk, the correlation coefficient was 0.76, which was also highly significant far beyond the 1% level. The correlation coefficient for the summer milk was 0.45, which was significant at the 1% level. These values were obtained when the figures followed by a question mark in table 2 were included in the calculations. The butterfat values below 2% are questioned, since the accuracy of the Babcock method at such levels is not as great as at the higher levels. For this reason these values were omitted in a recalculation, but this did not alter the correlation coefficients.

DISCUSSION

The reports in the literature have indicated considerable disagreement as to the presence of any esterified cholesterol in milk. The values for the esterified cholesterol range all the way from 100% (Wacker and Beck, '21) through progressively decreasing amounts (Denis and Minot, '18; Shope and Gowen, '28; Fox and Gardner, '24) to zero, in which case nothing but free cholesterol was present in the milk (Ansbacher and Supplee, '34; Dam, '34b; Mühlbock, '34). The latter work agrees with our results (table 2). As Ansbacher and Supplee ('34) have suggested, the earlier results in-

dicating the presence of ester cholesterol in milk are probably attributable to the analytical methods used.

All of the reports are in agreement that the total cholesterol in cows' milk ranges from about 9 to a maximum of 17 mg per 100 ml (table 5). This agreement occurs in spite of the fact that some of these workers maintained that there were large amounts of ester cholesterol in milk. For instance, Wacker and Beck ('21), who claimed that all of the cholesterol in milk was present in the ester form, found

TABLE 5
Cholesterol content of cows' milk as reported in the literature

AUTHORS	METHODS USED	FAT	CHOLESTEROL
		%	mg/100 ml
Denis and Minot ('18)	Colorimetric (Bloor)	3.2-5.0	10-17.6
Wacker and Beck ('21)	Digitonin precipitation (Windaus)	3.65	12.58
Nakanishi ¹ ('31)	—	3.85	12.67
Coccheri ¹ ('32)	—		12-15
Dam ('34)	Digitonin precipitation	3.65	13.36
		3.45	12.50
Mühlbock ('34)	Digitonin precipitation	1.80	9.18
Torrise ('40)	Liebermann. Burchard		10-12
Nataf-Mickelsen- Keys-Peterson ('48)	Schoenheimer and Sperry ('34)		
	Holstein	2.87	9.6
	Jersey	4.70	12.9
	Guernsey	3.98	11.5

¹ It was impossible to determine the methods used by Nakanishi and Coccheri.

a value of 12.6 mg total cholesterol, which is within the range reported by others. Since the ester cholesterol is usually determined as the difference between the free and the total, it would appear as though the earlier methods for measuring free cholesterol gave values that were too low.

There is some disagreement among the various workers in this field over the question of the relation between the percentage of fat and the cholesterol content of cows' milk. Denis and Minot ('18), who were the first to consider this question, claimed that the cholesterol showed a "direct and

proportional variation'' with the butterfat. This was based on a visual inspection of their data. The product-moment correlation coefficient for their values, however, is 0.19, which is insignificant. So in spite of their contentions to the contrary, their data show no relation between the butterfat and the cholesterol levels of milk. Fox and Gardner ('24), from a study of a small number of samples from a few cows, indicated that in certain cows "the cholesterol appears to follow approximately the output of fat." When they considered the over-all results of their small series, they decided there was no "exact ratio" between the butterfat and cholesterol content of milk. They quoted the results of Wacker and Beck ('21) to confirm their findings. More recently, Ansbacher and Supplee ('34) have claimed that "the cholesterol content of whole milk is quite variable and not dependent upon the fat content," but they gave exact data for only one sample of whole milk.

The results of the previous work are at variance with our own, in which a high correlation between the butterfat and the cholesterol content was found. It is possible that the earlier workers used too few samples to see the true relation between these two variables. For instance, the largest number of such samples used in earlier analyses was in the study of Denis and Minot ('18), in which 15 separate values for cholesterol and butterfat were reported. Another factor that may aid in explaining our finding of a high degree of dependence between these two values is the spread in the butterfat levels in the milk from the present cows. The lowest butterfat value in this series was slightly below 2%, while the highest was 6.2. This is twice the range found in Denis and Minot's study ('18).

None of the previous series has been extensive enough to permit an evaluation of the influence of season upon the cholesterol content of milk. Although we found a slight difference between the levels of cholesterol in summer and winter milk, this was not the same for the Holstein and Jersey cows. If this seasonal variation is of biological signif-

icance, it is doubtful whether it can be explained on the basis of dietary changes. The only significant difference in the rations of the two seasons was the addition of fresh pasture grass to a reduced winter ration during the summer. None of the materials fed to the cows in this experiment contained any cholesterol, since only plant products were used. Cholesterol is found in animal products (Okey, '45); plants contain other related sterols and at present it is questionable whether these are absorbed by mammals (Schoenheimer, '32). Dam ('34a) has shown that even when cholesterol in fairly large amounts was fed to a goat for three days, there

TABLE 6

Relation between the cholesterol content of blood and the cholesterol content of milk. (The blood and milk samples were secured May 31st)

COW		SERUM CHOLESTEROL		MILK CHOLESTEROL
		Free	Total	Total
		<i>mg/100 ml</i>		<i>mg/100 ml</i>
Holstein	838	24.1	183.1	7.0
Holstein	474	12.7	91.9	8.2
Holstein	480	19.4	152.5	11.1
Jersey	297	24.1	140.6	11.5
Guernsey	658	17.8	136.2	11.8
Guernsey	672	29.1	151.5	12.8
Jersey	275	19.8	134.4	13.3
Jersey	293	19.8	121.3	15.6
Guernsey	639	20.2	86.9	16.0

was no augmentation in the cholesterol content of the animal's milk.

Until now, there has been no systematic study of the cholesterol content of milk from different breeds. The present work indicates a significant breed difference in winter which is not apparent in summer. The explanation for this variation has been considered above.

The day-to-day variation in the butterfat content of milk has been studied by the earlier workers in this field. Some of the results (Eckles, '12, '20) were more extensive than the present ones and indicated a fairly high daily varia-

tion in this factor. There are no such previous studies on the variation in the cholesterol content of the milk. Our findings indicate that the day-to-day variation of the milk cholesterol is greater in summer than in winter. It is impossible to suggest any explanation for this difference.

In the early part of this study an attempt was made to see whether there was any relation between the cholesterol level in the blood and that in the milk from the same cows. The results of analyses of the milk and the jugular vein blood secured from the same cows on the same day are given in table 6. From these data it can be seen that there is no relation between the cholesterol content of the milk and of the serum. If Saarinen's ('44) work on the serum cholesterol as a precursor of butterfat is substantiated, it must involve a more complicated series of reactions than simple hydrolysis of the cholesterol esters and the transfer of either one or both of these components through the alveoli epithelium. The present results also show that the total cholesterol in the serum is not related to breed, and that the free cholesterol ranges from 12.7 to 23.2% of the total which is approximately the same, or at most only a slightly lower percentage than that in human beings (Sperry, '36).

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THE BASAL METABOLISM OF TWO HUNDRED AND EIGHTEEN GIRLS AND YOUNG WOMEN OF SOUTHERN ARIZONA, FOURTEEN TO TWENTY-THREE YEARS OF AGE, INCLUSIVE

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ONE FIGURE

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Basal energy metabolism of native white people living in this country has been shown by a number of investigators (Tilt and Walters, '35, in Florida; Eaton, '39, in New Orleans; and others) to be lower in subtropical climates than in temperate climates.

The state of Arizona, where the present study was made, has wide variations in its geography and climate. Fully two-thirds of the northern half consists of plains at high altitudes and mountainous areas. Much of this area is more than 4000 feet above sea level where the winters are cold and summers cool. Southern Arizona, on the contrary, presents a sharply different picture, especially in the south central and southwest. This area, covered by the valleys of the Salt, Gila, and Colorado rivers, is located in the area of the United States which has the most sunshine and the least relative humidity (Yearbook of Agriculture, '41).

Few studies of basal metabolism on girls who live in subtropical climates have been reported in the literature and there are none from the state of Arizona. Selection of subjects, therefore, was confined to girls living in southern Ari-

zona, where the work was being done. The age range selected includes the years from 14 to 23, inclusive.

EXPERIMENTAL

Subjects

Basal metabolisms have been determined on 218 subjects on the basis of 887 individual tests which were made at intervals during the years 1940 to 1947 in the winter months between October and April. All subjects were white and, with few exceptions, were born and reared in southern Arizona or arrived there in early infancy. The exceptions had lived in other states with similar climates for 5 years or less, several years previously. They were taken at random from volunteers in the junior and senior high schools and the State University of Arizona.

Before being accepted as subjects they were questioned as to the possibility of their having had thyroid therapy, since we have observed that thyroid extract is administered frequently in this part of the country. Tests were made during the menstrual cycle, provided the subject was rested and relaxed.

Procedure

Using the indirect method of calorimetry, two 8- to 10-minute tests were made on each of two or more mornings, following each other as closely as possible. No preliminary tests were made. Subjects returned for further testing until oxygen consumption in cubic centimeters per minute from either of the tests on a given day agreed within 5% with the consumption on another day. Surface area was calculated from the height-weight formula of DuBois as cited by Carpenter ('39). Calories per m^2 per hour were calculated by each of 4 methods commonly used in evaluation of basal metabolism. These are: (1) average of results of all tests within 5% of 1 accepted as a base line on at least two mornings, (2) average of all accepted tests, (3) accepted first tests only, (4) average of accepted tests on the first morning only.

RESULTS

Physical data

Physical data expressed as yearly means are shown in table 1. Some cases deviated in weight more than minus 10 and plus 15% from the Wood standards as cited by MacLeod and Taylor ('44). Several of these cases, when measured by the Pryor standards ('40) which take into consideration skeletal structure, fell within a normal range. Only a few appeared to be overweight, although several were quite slender. The range of oral temperatures was 96.3° to 98.6°F. There was a wide range of recumbent pulses per minute: 44 to 85.

TABLE 1

Physical data of subjects

CASES	AGE YEARLY MEAN	WEIGHT	HEIGHT	SURFACE AREA	PULSE PER MIN. AVE.	TEMP.	VITAL CAPACITY DEV.	CALORIES PER M ² PER HR.
		<i>kg</i>	<i>cm</i>	<i>m²</i>		<i>° F.</i>	<i>%</i>	
23	14.48	52.6	162.6	1.55	65	97.9	7.9	36.22
23	15.45	54.2	165.4	1.59	66	98.3	9.0	34.37
24	16.53	55.3	165.9	1.62	64	98.1	8.3	33.12
25	17.51	56.6	166.4	1.63	66	97.9	5.5	32.12
36	18.53	55.6	164.6	1.60	62	97.8	9.4	31.21
21	19.33	56.4	166.8	1.62	64	97.7	10.2	31.12
24	20.52	57.2	167.1	1.64	62	97.8	9.5	31.14
21	21.39	60.3	166.5	1.67	62	97.7	11.2	31.30
12	22.31	57.0	167.6	1.64	62	97.3	9.2	30.84
9	23.48	60.9	166.1	1.68	59	97.7	11.5	30.79

The respective coefficients of variation of vital capacity for each year are large: 12.2, 12.7, 11.5, 12.3, 13.9, 13.0, 13.4, 16.7, 17.8, and 18.7%. Vital capacity was referred to 3 commonly used standards: (1) Dreyer ('19), based on weight; (2) West ('20) referred to surface area, (3) West ('20) based on height. Although differences are small in some years, for each year except the 23rd West's standard referred to surface area, without regard to plus or minus sign, predicted the actual vital capacity more closely than his standard referred to height. This standard also predicted more closely

than Dreyer's standard referred to weight for each year except the 15th and 20th. Mean deviations from West's standard based on surface area, without regard to algebraic sign, are therefore included in table 1.

Statistical treatment of data

Calories in table 1 are based upon the first method of evaluating data. By this method only 14% (31 cases) of the total returned on a third morning, and 2% (4 cases) on a 4th. In only 10% (21 cases) were both tests on first mornings omitted. But 50% (109 cases) of first tests on the first morning could not be included because 80 were more than 5% above any other test, 9 below, and the spirograms of the other 20 too irregular to use.

Smoothed means of the first 5 years for each of the 4 methods of evaluating data from individual tests were obtained by reduction of data to linear form (Yule and Kendall, '45). Logarithms of calories minus a constant for each year were plotted, and straight lines fitted by means of the equation $\log (y-c) = \log a \cdot x (\log b)$. Y represents calories per square meter per hour; c, a constant; x, age in years; log a, the (y-c) intercept at 0 years; and log b the numerical value of the slope or yearly drop in calories. The constant used in the first method was 29.5, and in each of the other methods. 30.

Table 2 shows the smoothed means by each of the 4 methods for the years 14 to 18, inclusive, and true means for the years 19 to 23. When the data of the first 5 years were reduced to linear form, the best straight line fit was obtained by the second method; the next by the first. As was to be expected, the lowest yearly means were obtained by the first method. A sharp drop in calories was shown by all methods, the means in the first changing from 36.40 to 31.21 during the 5 years.

The coefficients of correlation with age of the weighted smoothed means are highly significant by each of the first two methods: i.e., -0.995 ± 0.0011 and -0.997 ± 0.0010 , respec-

TABLE 2

Means in calories per m² per hour and variability by 4 methods of evaluating data

AGE, YRS.	CASES	CAL.	σ^1	C.V. ²	CASES	CAL.	σ^1	C.V. ²
		<i>Five % check</i>				<i>All tests averaged</i>		
14.00-14.99 14.48 (M)	23	36.40	3.32	9.12	23	36.78	3.34	9.07
15.00-15.99 15.45	23	34.37	2.64	7.67	23	34.71	2.45	7.04
16.00-16.99 16.53	24	32.94	2.15	6.52	24	33.27	1.95	5.85
17.00-17.99 17.51	25	31.92	3.24	10.14	25	32.27	2.50	7.76
18.00-18.99 18.53	36	31.21	2.08	6.68	36	31.58	2.06	6.51
19.00-19.99 19.33 (M)	21	31.12	2.45	7.87	21	31.40	2.56	8.15
20.00-20.99 20.52	24	31.14	2.99	9.60	24	31.67	2.95	9.31
21.00-21.99 21.39	21	31.30	2.26	7.22	21	31.67	2.38	7.52
22.00-22.99 22.31	12	30.84	2.37	7.68	12	31.29	2.60	8.31
23.00-23.99 23.48	9	30.79	1.32	4.29	9	31.62	1.18	3.73
		<i>1st test</i>				<i>1st morning</i>		
14.00-14.99 14.48 (M)	22	38.40	4.03	10.48	22	36.56	2.86	7.81
15.00-15.99 15.45	21	36.16	3.06	8.45	22	34.70	2.80	8.07
16.00-16.99 16.53	24	34.52	2.55	7.39	24	33.37	2.35	7.05
17.00-17.99 17.51	21	33.31	3.78	11.36	24	32.41	3.15	9.72
18.00-18.99 18.53	34	32.43	2.38	7.34	36	31.73	2.02	6.36
19.00-19.99 19.33M	17	31.00	2.31	7.45	21	31.51	2.35	7.46
20.00-20.99 20.52	18	32.58	3.23	9.91	24	32.45	3.23	9.95
21.00-21.99 21.39	17	32.56	2.74	8.42	21	31.78	2.32	7.30
22.00-22.99 22.31	12	31.79	3.31	10.41	12	31.52	2.91	9.23
23.00-23.99 23.48	8	31.30	3.44	10.99	9	32.33	1.82	5.63

¹ Standard deviation.² Coefficient of variation.

tively. By the third and 4th methods they are just barely significant, i.e., -0.929 ± 0.0080 and -0.953 ± 0.0542 , respectively.

No real differences are found in the yearly means from 19 to 23 by either of the first two methods. However, by the third and 4th methods approximately half of the differences show a better than even chance that they are real. When the averages of these yearly means by each of the first two methods are compared with the means of the 18th year in each case, no real differences are found. Lumped means of 31.1 and 31.5 calories, respectively, have been taken, therefore, for the 18th to the 23rd year for each of the two methods.

DISCUSSION

Of the 218 subjects, only 31 had to return a third time to obtain the 5% agreement and 4 a 4th time. If the data of the first 5 years by the second method had been based upon the average of only the first two mornings for all subjects, instead of including more than two mornings as was done for 17 of the subjects, the smoothed means would have been increased a small amount for each year; the differences, being less than $\frac{1}{2}$ calorie in each case, are not statistically significant. The coefficient of correlation with age would remain unchanged; i.e., highly significant. Individual basal metabolisms would, however, have been significantly altered in some cases, 6 of the 17 cases which were recalculated for this purpose changing to levels which showed large differences.

When the smoothed means for each of the first 5 years, as obtained by the 2nd, 3rd, and 4th methods, are compared with those of the first, which is the lowest in each case, only the third shows a real difference for each of the 5 years, the results by the third method being higher in each case. The second showed no statistically significant difference for any of the years and the 4th none for the first 4 years, only the 18th year showing a better than even chance that the difference is real. Variability around the means is less by the second

method, as is demonstrated by the coefficients of variation for each year.

It would seem from the above that, for results obtained by the method of age-selected averages used here, the first two methods of evaluating individual tests would be most suitable in establishing basal metabolism standards. These data, at the time of writing, are being further analyzed on the basis of physical developmental levels and basal calories per day irrespective of age classification and independent of the method of averages (Wetzel, '44).

Comparison with other studies

Although methods used in determining basal metabolism are not the same in all studies, nevertheless some comparisons seem to be justified. As may be observed in figure 1, the basal metabolism of the younger subjects in the present study is fairly close to that of some of the studies reported from higher latitudes, but beginning at about the middle of the 15th year

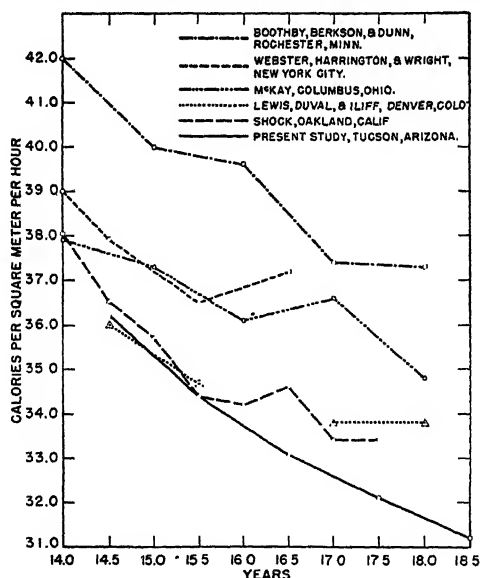


Fig. 1 True yearly means of the present study, as calculated by the first method of evaluating data, compared with other studies.

it rapidly approaches a much lower level. This is clearly demonstrated when comparison is made with the study of Shock ('42), whose subjects lived in Oakland, California. Naldandov, Heller, Krause and Purdy ('38), working in Oklahoma, reported that their younger girls had a rate of metabolism as high as those of the same age in the north, but that as they grew older Oklahoma girls showed a much sharper drop than northern girls.

Basal metabolism as reported from Florida by Tilt and Walters ('35) for girls 19 years of age and older is strikingly similar to that of southern Arizona for subjects of the same age, calories per square meter per hour of the former being 30.5, 31.4, 32.1, and 32.6 for the years 19, 20, 21, and 23, respectively. The study in the north which reported the largest number of cases is that of Boothby, Berkson and Dunn ('36). Using standards known as the Mayo Foundation standards which are based on first tests only, they predict 36.74 calories per m^2 per hour for the 19th year of age and 36.18 calories for the years 20 to 24, inclusive. In the present study, means by the method of first tests only are 31.00 calories for the 19th year of age and 32.06 calories for the age range of 20 to 23 years, inclusive.

Effect of environmental temperature

Five cooperating state universities have reported a study of the basal metabolism of a large number of college girls (Pittman et al., '43). In table 3 of the present study we have shown these states in the order of increasing mean annual temperatures (Yearbook of Agriculture, '41), together with the mean basal metabolisms as reported in each case for the 18-year-old groups.

Although temperature is only one factor to be considered in a study of the effect of climate, it is of interest to note that these states, with the exception of Oklahoma which has a limited number of cases, show a definite trend toward a diminishing basal level with increasing temperature.

The attainment of this low adult value beginning at the 18th year would seem to indicate the appearance of the chemical regulation mechanism in women in warm environments which was demonstrated by Hardy and DuBois ('40) and Hardy, Milhorat and DuBois ('41). These workers, studying the basal metabolism of 8 adult women in a room calorimeter with the direct method of calorimetry and a carefully regulated

TABLE 3

Basal metabolism of 18-year-old girls in midwestern states and in the present study

STATE	CASES	MEAN ANNUAL TEMPERATURE (1934-38 INC.)	CALORIES PER M ² PER HOUR
Minnesota	34	41.6	35.7
Iowa	31	49.5	35.1
Ohio	40	52.0	33.8
Kansas	58	56.6	33.6
Oklahoma	19	61.7	34.0
Arizona, southern	36	67.2	31.2

temperature index (controlled barometric pressure, humidity, air movement, and environmental temperature), demonstrated the existence of a zone of minimal metabolism between 27°C. and 32°C. (81°F. and 90°F.). They reported a low mean of 30.9 calories per m² per hour in this 5-degree temperature range after an adaptation period of from two to three hours.

Effect of altitude

Although Tucson has an elevation of 2400 feet above sea level, it would seem improbable, from the results of the present study and a review of the literature, that altitude influences the basal metabolism provided one can discuss it properly separated from other factors contributing to the climatic effect. The studies of Lewis, Duval and Iliff ('43) support this observation. As shown in figure 1, Shock, working at the low altitude of Oakland, California, found higher mean values than those reported by us, and higher also than Lewis' 14-

and 15-year-old group studied at a much higher altitude in Denver, Colorado.

SUMMARY

1. Basal metabolism determinations have been made on 218 girls and young women of southern Arizona from 14 to 23 years of age, inclusive.

2. Calories per m^2 per hour were calculated by 4 methods of evaluating individual tests: (1) average of results of all tests within 5% of one accepted as a base line on at least two mornings, (2) average of all tests, (3) first tests only, (4) average of tests on first mornings only.

3. Smoothed yearly means in calories dropped sharply by all methods, by the first method from 36.40 to 31.21 for the years from 14 to 18, inclusive. Correlation with age was found to be highly significant statistically by the first two methods and less significant by the other two. When reducing the true yearly means to linear form, it was found that of the 4 methods of evaluating individual tests the best straight line fit was obtained by means of the second method and the next by the first. When the respective yearly means obtained by each of these two methods were compared, no statistically significant differences were found although those by the first were lower in each case. Variability around the means was less by the second method.

4. Lumped means of 31.1 and 31.5 calories were taken for the first two methods, respectively, for the years 18 to 23, inclusive. This low energy level would seem to indicate the appearance in women at this time of the zone of minimal metabolism demonstrated by Hardy, Milhorat and DuBois ('41).

5. When the 18-year-old group was compared with similar groups of the same age reported from 5 midwestern states, a diminishing metabolism was observed with increasing mean annual temperature.

6. The present study was made at an altitude of 2400 feet. If altitude per se tends to increase basal metabolism, as some

investigators have claimed, then the present low level of metabolism should not have been observed. Therefore our data do not support this view.

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MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1949 Award of \$1000, established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the spring of 1949.

The Award will be given to the laboratory (non-clinical) or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year, January 1 to December 31, the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1948 must be in the hands of the Chairman of the Nominating Committee by January 15, 1949. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

HAROLD H. WILLIAMS
Cornell University, Ithaca, N. Y.

CHAIRMAN, NOMINATING COMMITTEE

BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1949 Award of \$1000, and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the judges may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1949. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 15, 1949. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

JAMES M. ORTEN
*College of Medicine,
Wayne University,
Detroit, Michigan*

CHAIRMAN, NOMINATING COMMITTEE

OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of \$1000, established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1949 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 15, 1949.

D. W. WOOLLEY

*Rockefeller Institute for
Medical Research, New York, N. Y.*

CHAIRMAN, NOMINATING COMMITTEE

EFFECTS OF AUTOXIDATION ON ANTIACRODYNIC POTENCY OF FATS AND LINOLEIC ESTERS ¹

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TWO FIGURES

(Received for publication May 29, 1948)

Studies on the nutritive value of rancid fats have been carried out under two types of dietary regimes. One, by Burr and Barnes ('43), involved the ability of a rancidified high fat diet to sustain growth. The other, by Quackenbush ('45), involved the ability of small amounts of rancidified fatty acids to cure acrodynia. In the latter case, peroxide values were used as an indicator of the state of oxidation. However, Farmer ('46) has pointed out that peroxides disappear as oxidation and polymerization proceed. High peroxide values therefore do not necessarily reflect the most advanced stages of oxidation.

In a recent review Quackenbush ('45) stated that the literature contained a number of reports of malnutrition resulting from rancid dietary fat. In some instances the malnutrition has been traced to the destruction of dietary essentials such as the fat-soluble vitamins (Quackenbush, Cox and Steenbock, '42) and water-soluble vitamins (Pavcek and Shull, '42).

¹ This research was undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the War Department.

² Contribution No. 363 from the Department of Chemistry, Kansas State College, Manhattan.

However, Quackenbush ('45) has stated that until experimental evidence provides the full explanation it must be assumed that rancid fats are able to exert a direct toxic effect. As increasing quantities of beef, pork, and poultry are being kept in cold storage, it might be desirable to know whether the rancid fats which are formed during this storage period (Schrieber et al., '47; Wagoner et al., '47) lose nutritional value.

In the present study, the ability of small quantities of oxidized fats and fatty acids to cure acrodynia was used as a means of testing their nutritive value. As pyridoxine and pantothenic acid (Birch, '38; Quackenbush, Steenbock, Kummerow and Platz, '42), and tocopherol (Hove and Harris, '46) have been found to be functional in fat deficiency, these dietary factors were fed to some of the rats in the various groups. Furthermore, the effects of these factors and the effects of oxidized fats on the characteristics of the mixed fatty acids deposited in the tissues were determined by means of spectrophotometric analysis.

MATERIALS AND METHODS

Preparation of materials

The ethyl and methyl linoleate were prepared from corn oil³ via the tetrabromide according to the method of Rollet ('09), distilled under high vacuum and kept under an atmosphere of nitrogen at -13°C . The various esters listed in table 1 were prepared as follows: The rancidified methyl linoleate of high peroxide value was prepared by bubbling tank oxygen through 10 gm of the freshly distilled ester at 80°C . for three hours. The drastically oxidized ethyl ester was prepared by bubbling tank oxygen through the free acid for 20 instead of 3 hours. This acid was then esterified in absolute ethyl alcohol. This resulting material was extracted with Skellysolve F, washed with water, dried over sodium sulphate and

³ Furnished through the courtesy of Dr. R. A. Baldwin, Corn Products Refining Co., Argo, Illinois.

freed from solvent; on standing, the product gradually became viscous in character.

The alkali-conjugated ethyl linoleate was prepared from freshly-distilled ethyl linoleate. The ester was isomerized with potassium hydroxide in ethylene glycol at 180°C. for 30 minutes (Mitchell, Kraybill and Zscheile, '43), poured over chipped ice and acidified with concentrated hydrochloric acid. The resulting mixture of conjugate isomers was then converted to the ethyl esters. The 10, 12 ethyl linoleate was prepared from dehydrated castor oil⁴ by the method of Von Mikusch ('42). The 10, 12 acid was isolated from the mixed

TABLE 1
Characteristics of supplements

SUPPLEMENT	AMOUNT FED DAILY	IODINE VALUE	PEROXIDE VALUE	SPECIFIC ABSORPTION COEFFICIENT AT 2840 Å
	<i>mg</i>			
Fresh ethyl linoleate	25	167	9	0.3
Rancid methyl linoleate	25	109	629	0.6
Drast. oxid. ethyl linoleate	25	49	76	2.3
Conjugated ethyl linoleate	25	145	5	84.6
10, 12 ethyl linoleate	25	115	37	105.0
Rancid turkey fat	110	79	63	1.4
Fresh turkey fat	110	78	5	0.3

fatty acids by repeated fractional crystallization from Skellysolve F, ethyl alcohol and diethyl ether. The resulting product, which had a melting point of 55–56°C., was then converted to the ethyl ester. The rancid turkey fat was extracted from the skin of turkeys which had been kept in cold storage for two years; the fresh fat was extracted from the skin of freshly-killed birds. The skin was extracted with Skellysolve F, and the extract washed with water, dried over sodium sulphate and freed from solvent. The birds had been broad-breasted bronze turkeys of the same strain and had been kept on a standard turkey ration (Kummerow et al.,

⁴ Furnished through the courtesy of O. Eisenschiml, Scientific Oil Compounding Co., Chicago.

'48). In each case the skin fats contained approximately the same percentage of mixed fatty acids, i. e. 20% linoleic, 50% oleic, 30% saturated, 0.1–0.5% arachidonic and no linolenic acid.

The various preparations were kept at -13°C . under nitrogen except during use. They were fed to the rats by means of a calibrated medicine dropper.

Preparation of animals

The diet and preparation of the animals were similar to those described by Quackenbush et al. ('39).

The diet was prepared as follows: 200 mg thiamine hydrochloride and 400 mg of riboflavin were dissolved in N/50 acetic acid, mixed with 18 pounds of casein⁵, dried and then mixed with 78 pounds of glucose (cerelose) and 4 pounds of Wesson salts in a standard feed mixer. The animals⁶ were supplemented once a week with one drop of fish liver oil⁷ containing 250 I.U. of vitamin A⁸ and 100 I.U. of vitamin D⁹ per drop.

On this diet animals with an initial weight of 40–50 gm developed an acute acrodynia in 7–8 weeks. The severity of the acrodynia was recorded as a dermal index (Quackenbush et al., '39). This index was a numerical expression of the stage of development of lesions in the lips, eyelids, forepaws, hindpaws, ears and tail, and increased with the severity of the acrodynia.

Thirteen groups of three rats each were fed the supplements listed in table 2 for a three-week period. The rats were then killed, and each group saponified with 30% aqueous

⁵ Vitamin test casein, purchased from General Biochemicals, Inc., Chagrin Falls, Ohio.

⁶ Three weeks of age, obtained from Sprague-Dawley and Co., Madison, Wisconsin.

⁷ Residue oil obtained on molecular distillation.

⁸ Concentrate, obtained through the courtesy of Dr. Hickman, Distillation Products, Inc., Rochester.

⁹ Crystalline, obtained from Dr. J. Waddell, E. I. du Pont de Nemours and Co., New Brunswick.

TABLE 2
Effects of fat supplements on dermal lesions and carcass fat

GROUP	FAT SUPPLEMENTS	MEAN BODY WEIGHT CHANGE IN 3 WEEKS	DERMAL INDEX		EFFECT ON DERMAL LESIONS	MIXED FATTY ACIDS		IODINE VALUE
			Initial	3 wks.		Mean weight	%	
<i>I. Without pyridoxine and calcium pantothenate</i>								
1	None	-6.5	5.0	6.0	Negative	0.77	1.3	86.8
2	Conjug. et. linoleate	+1.7	5.7	3.0	Improvement	1.40	2.0	77.9
3	Drast. oxid. et. linoleate	-9.0	4.0	7.0	Negative	0.85	1.4	93.5
4	10, 12 ethyl linoleate	-11.3	4.0	7.3	Negative	1.58	2.0	85.6
5	Fresh turkey fat	+6.7	5.0	2.0	Curative	1.47	2.2	82.2
6	Rancid turkey fat	+4.0	4.3	2.7	Improvement	1.87	2.5	83.9
7	Fresh me. linoleate	+7.0	4.4	2.0	Curative	1.46	2.3	86.4
8	Rancid me. linoleate	0.0	4.5	2.5	Improvement	1.50	2.1	77.8
9	Rancid me. linoleate + tocopherol ¹	-1.0	4.0	3.0	Improvement	2.14	3.0	80.3
10	Rancid me. linoleate + ethanalamine ²	-9.0	5.0	5.7	Negative	1.46	1.9	84.7
<i>II. With 20 µg pyridoxine and 50 µg cal. pantothenate daily</i>								
11	None	+43.0	4.3	4.3	Improvement	3.54	3.0	70.6
12	Rancid turkey fat	+68.0	4.3	1.3	Curative	12.23	9.0	64.8
13	Rancid methyl linoleate	+35.3	4.7	1.7	Curative	5.09	4.5	69.9
14	10, 12 ethyl linoleate	+33.5	4.7	3.4	Improvement	7.02	7.2	65.6

¹ Tocopherol — 14.0 mg per week.

² Ethanalamine — 18.0 mg daily.

alkali as described by Quackenbush and Steenbock ('42). The hydrolysate was freed of non-saponifiable material by extraction with Skelly-solve F and acidified with diluted hydrochloric acid (1:1). The crude fatty acids were extracted with Skelly-solve F, washed thoroughly with water, dried over sodium sulphate, freed from solvent and weighed. Io-

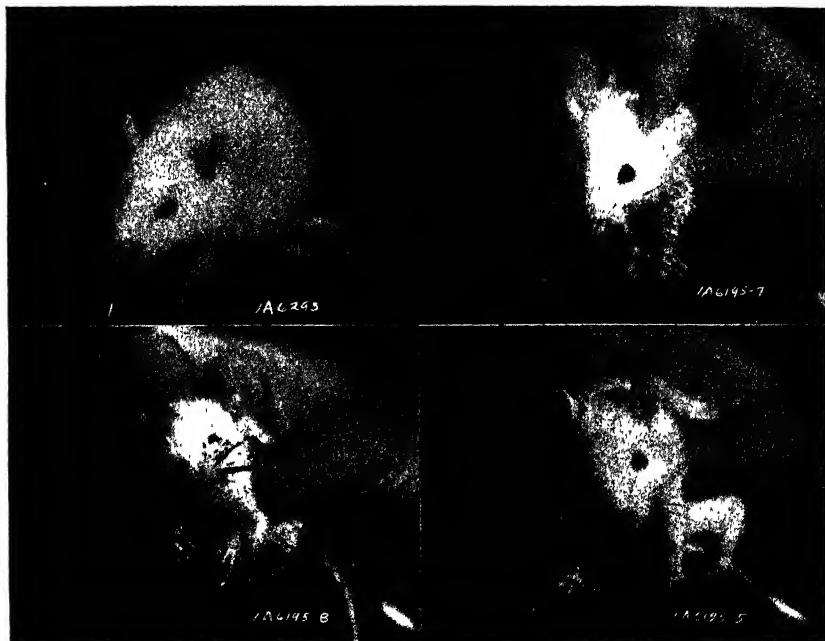


Fig. 1 Control 1A6293; rancid methyl linoleate 1A6195-7; 10, 12 ethyl linoleate 1A6195-8 and rancid methyl linoleate plus pyridoxine and calcium pantothenate 1A6195-5.

dine values were determined according to the method of Yasuda ('31) and the composition of the mixed fatty acids by the spectrophotometric method of Brice et al. ('45).

EXPERIMENTAL RESULTS

Effects on acrodynia

The results indicated that drastically oxidized or 10, 12 ethyl linoleate were not effective curative agents (table 2).

In fact, a more acute acrodynia and a greater decrease in body weight were noted in the animals of these groups than in those which had received no supplement (fig. 1). The alkali-conjugated ethyl linoleate, the rancid methyl linoleate of high peroxide value and rancid turkey fat alleviated the symptoms but were not so effective as the freshly-distilled

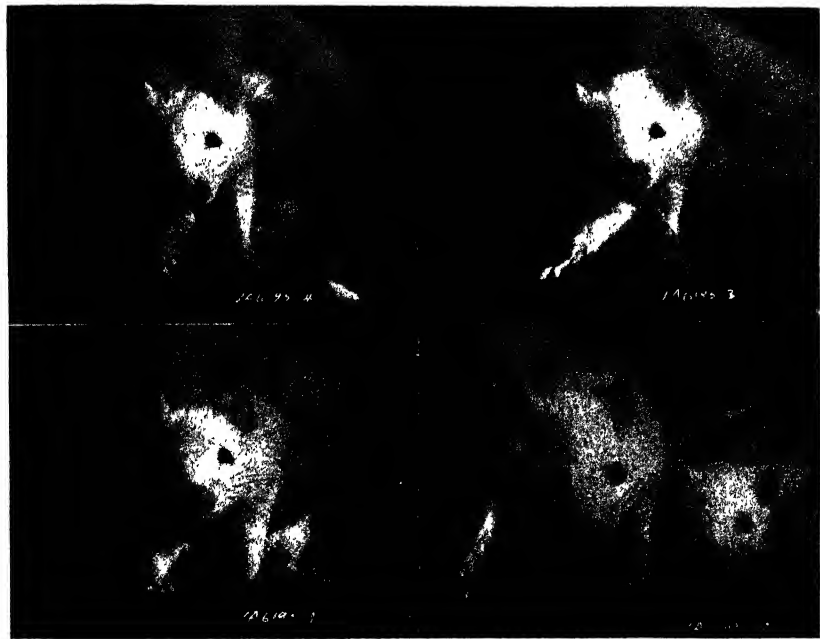


Fig. 2 Fresh turkey fat 1A6195-4; rancid turkey fat 1A6195-3; rancid turkey fat plus pyridoxine and calcium pantothenate 1A6195-1; rancid turkey fat (right) and rancid turkey fat plus pyridoxine and calcium pantothenate (left) 1A6195-12.

ester or fresh turkey fat (fig. 2). Topocopherol did not improve the effectiveness of rancid ethyl linoleate, and ethanollamine aggravated the symptoms.

When pyridoxine and calcium pantothenate were fed in addition to rancid methyl linoleate, the dermal symptoms were alleviated. However, the animals on these rations gained less weight than those whose diets had been supplemented with only pyridoxine and calcium pantothenate (table

2). This growth-depressing action of rancid methyl linoleate was also noted by Gyorgy et al. ('42). Pyridoxine and calcium pantothenate added to the curative value of rancid turkey fat but had little effect on the curative value of 10, 12 ethyl linoleate.

Effects on characteristics of extracted fatty acids

The presence of pyridoxine and calcium pantothenate, rather than the presence of rancid or fresh fat, seemed to have the most influence on the quantity and character of the fat deposited (table 2). Animals fed pyridoxine and calcium pantothenate in addition to rancid fat deposited from two to three times more fat than those which had received only rancid fat, or 4.5–9.0 and 2.0–2.5% respectively. Except for the animals which had been fed drastically oxidized ethyl linoleate, the percentage of fat extracted from the animals fed fresh or rancid fat varied only 0.5%, or from 2.0–2.5%. The drastically oxidized linoleate was probably not absorbed readily from the intestinal tract, as these animals contained only 0.2% more fat than those which had received no supplement.

The iodine values of the fats decreased from 8 to 20 points when pyridoxine and calcium pantothenate were fed in addition to fat (table 2). The fat extracted from the animals which had received no fat supplements or drastically oxidized ethyl linoleate had the highest iodine value, or 86.8 and 93.5 respectively. Those which had received pyridoxine and calcium pantothenate in addition to rancid turkey fat or 10, 12 ethyl linoleate had the lowest iodine value, or 64.8 and 65.6, respectively.

Spectrophotometric analyses of the mixed fatty acids indicated that the percentages of essential fatty acids in the tissues were not adversely affected by the feeding of rancid fat (table 3). The animals which had been fed only rancid turkey fat contained the highest, and those which had received no supplement contained the lowest, percentages of linoleic acid, or 7.2 and 4.0%, respectively.

TABLE 3

Spectrophotometric studies of mixed fatty acids from carcasses of rats fed various fats

GROUP	SUPPLEMENT	SATURATED		OLEIC		LINOLEIC		ARACHIDONIC	
		%	mg ¹	%	mg ¹	%	mg ¹	%	mg ¹
<i>I. Without pyridoxine and calcium pantothenate</i>									
1	None	22.9	295	67.5	870	4.0	52	5.6	72
2	Conj. ct. linoleate	33.8	675	54.8	1096	6.3	126	5.1	102
3	Drast. oxid. et. linoleate	20.5	287	67.3	942	5.1	71	7.1	99
4	10, 12 et. linoleate	24.0	480	65.1	1301	6.2	124	4.7	94
5	Fresh turkey fat	27.5	605	61.8	1360	6.1	134	4.6	101
6	Rancid turkey fat	24.9	623	63.9	1597	7.2	180	4.0	100
7	Fresh me. linoleate	25.5	586	62.5	1433	6.5	149	5.5	127
8	Rancid me. linoleate	33.7	707	55.8	1172	4.8	101	5.7	120
9	Rancid me. linoleate + tocopherol	26.0	780	65.3	1960	4.8	144	3.9	117
10	Rancid me. linoleate + ethanolamine	23.4	445	66.5	1263	5.8	110	4.3	82
<i>II. With pyridoxine and calcium pantothenate</i>									
11	None	27.8	833	68.5	2055	2.0	60	1.0	30
12	Rancid turkey fat	34.8	3130	60.9	5475	2.6	234	1.1	99
13	Rancid me. linoleate	28.5	1282	68.0	3060	2.0	90	1.5	67
14	10, 12 et. linoleate	21.2	1527	76.1	5480	1.4	101	0.9	64

¹ Mean per 100 gm of carcass. No linolenic acid was present except for traces in groups which received pyridoxine and calcium pantothenate.

Less than a 3.5% variation between groups was noted in the total linoleic and arachidonic acid content. The animals which had been supplemented with drastically oxidized ethyl linoleate contained the highest, and those supplemented with rancid ethyl linoleate contained the lowest, percentage of total essential fatty acids, or 12.2 and 8.7% respectively. When pyridoxine and calcium pantothenate were also fed, the total essential fatty acids varied from 2.3 to 3.7%. The total percentage was therefore only one-third as large as in the absence of pyridoxine and calcium pantothenate. However, comparisons based on percentages alone might be misleading, as the animals which had received pyridoxine and calcium pantothenate had greatly increased in weight. Therefore, comparisons based on the mean weight of fatty acid per 100 gm of tissue were made, in order to reflect differences between groups more accurately.

From these data it can be seen that in every case the animals which had received pyridoxine and calcium pantothenate synthesized substantial amounts of oleic and saturated fatty acids. Furthermore, the animals which had received rancid methyl or 10, 12 ethyl linoleate as well as pyridoxine and calcium pantothenate contained from 1,005 to 3,425 mg more oleic and 449 to 694 mg more saturated fatty acid than the control groups. The quantity of total essential fatty acids was not increased even though approximately 500 mg of linoleate was fed to these animals during the assay period. It is possible that pyridoxine or calcium pantothenate served as biological antioxidants or helped to convert rancid or conjugated acid to oleic and saturated fatty acid.

DISCUSSION

The nutritive value of rancid fats was improved when the diets of the experimental animals were supplemented with pyridoxine and calcium pantothenate. As yeast contains these two vitamins, its addition to the dietary regime (Burr and Barnes, '43) could influence the ability of a rancidified high fat diet to sustain growth. Whipple ('33), for example, has

shown that rats fed oxidized lard resumed growth when their diets were supplemented with additional quantities of ether-extracted yeast. It is interesting to note, however, that in the present study it was found that 10, 12 ethyl linoleate was not curative in the presence of pyridoxine and calcium pantothenate. Burr ('42) has shown that 9, 11 linoleic acid also lacks curative properties under these conditions.

Farmer ('46) and others have shown that in the first stages of autoxidation of linoleic acid the double bonds are conjugated with the simultaneous formation of hydroperoxides. During the later stages of autoxidation over 70% of the double bonds have been reported to be conjugated (Bolland and Koch, '45). In agreement with Burr ('42), the present study indicates that fats in the first stages of autoxidation alleviated the symptoms, but were not so effective as fresh fats. Fats in the later stages of autoxidation as represented by 10, 12 and drastically oxidized ethyl linoleate respectively were non-curative.

The various supplements of linoleic acids seemed to have been metabolized or converted to oleic and saturated fatty acids, although less fresh than rancid or 10, 12 linoleate was converted. When pyridoxine and calcium pantothenate were fed in addition to fat supplements, the oleic and saturated fatty acid contents of the tissues were greatly increased. However, these fatty acids must have been synthesized from carbohydrate or protein, as increased amounts of them were also found in the animals which had received no fat supplement.

It is possible that the availability of pyridoxine and pantothenic acid is a limiting factor in rat acrodynia. Umbreit and Gunsalus ('45) have shown that pyridoxine functions in a coenzyme system in amino acid metabolism. When pyridoxine is present in suboptimal amounts, it may not be able to function in both the metabolism of unsaturated fatty acids and the building of protein. The present study indicates that in extreme cases undesirable fats seem to have a priority claim,

so to speak, on the pyridoxine already stored in the tissues, as the animal actually lost weight and the symptoms were aggravated. Whether pantothenic acid is also necessary was not brought out in this investigation. However, Quackenbush, Steenbock, Kummerow and Platz ('42) indicated that both pyridoxine and pantothenic acid are functional in rat acrodynia.

The high peroxide value and the small amount of conjugated fat in the rancid turkey fat indicate that only the first stages of oxidation are involved during the cold storage of poultry. The rejection by a taste panel of cooked meat which contained rancid fat (Kummerow et al., '48) may have had a physiological basis. However, as the stored meat probably contained substantial amounts of pyridoxine and pantothenic acid, the present data indicate that ingestion should have caused no harmful effects.

SUMMARY

The ability of small quantities of oxidized fats to cure acrodynia was used as a means of testing their nutritive value. The results indicated that rancid methyl linoleate or the fat extracted from turkeys which had been subjected to cold storage for two years alleviated the symptoms but were not as effective as the freshly distilled ester or fresh turkey fat. Tocopherol did not improve the effectiveness of rancid ethyl linoleate and ethanolamine aggravated the symptoms. Drastically oxidized fats or 10, 12 ethyl linoleate were not effective curative agents.

When pyridoxine and calcium pantothenate were fed in addition to rancid fat the dermal symptoms were alleviated. This response was not due to pyridoxine or calcium pantothenate alone, but to a combination of the fat- and water-soluble factors. Spectrophotometric analysis of the carcass fats indicated that the various supplements of oxidized linoleic esters were converted to oleic and saturated fatty acids. Pyridoxine and calcium pantothenate seemed to be functional in this process.

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THE NUTRITIVE VALUE OF SOME LEGUMES.
LATHYRISM IN THE RAT. THE SWEET PEA
(*LATHYRUS ODORATUS*), *LATHYRUS SATIVUS*,
LATHYRUS CICERA AND SOME OTHER SPECIES
OF *LATHYRUS*¹

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ONE FIGURE

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The toxicity of certain species of *Lathyrus* and related legumes for man and many domestic animals has been recognized since early in the eighteenth century (Schuchardt, 1886-87). Outbreaks of lathyrism in man in India have been studied extensively by Stockman ('29, '31, '34a, '34b), and Shourie ('45), and in Spain by Díaz and his collaborators ('41, '42, '43), who have investigated lathyrism in that country from both the clinical and experimental viewpoints. Important in the production of lathyrism in man are *Lathyrus sativus*, *clymenum*, and *cicera*. The peas, which are usually eaten by the poorer classes in India and Spain only when the supply of cereal grains is deficient, must constitute a significant part of the diet, one-third to one-half, according to Stockman, ('29), in order to cause the disease. Males are more susceptible than females, an incidence of 20 males to one female be-

¹Grants in aid from the Nutrition Foundation of New York and the Faculty Research Fund of the Horace H. Rackham School of Graduate Studies of the University of Michigan have made possible this and related studies of the nutritive value of peas of the genus *Lathyrus*.

ing reported in one community by Díaz ('41). The symptoms (notably muscular weakness and paralysis of the extremities) have suggested to many the presence of a neurotoxin which may cause a degeneration of the spinal cord. The pathological study of a case of human lathyrism (*L. sativus*) has shown severe injury to the nervous system (Filimonoff, '26).

Marked variations in the susceptibility of domestic animals to *Lathyrus* pea intoxication have been observed (Stockman, '29). It is usually stated that lathyrism (*Lathyrus sativus* and *cicera*) cannot be produced experimentally in the white rat (Visco, '23, '24a, '24b; McCarrison, '28; Zagami, '31; McCarrison and Krishnan, '34; Díaz and Vivanco, '42). Geiger, Steenbock, and Parsons ('33), however, observed lathyrism in the rat after the flowering sweet pea (*Lathyrus odoratus*) was fed in considerable amounts. This species of *Lathyrus* has also been associated with human lathyrism in Spain (Díaz, '41). The data on the nutritive properties of *Lathyrus* species are somewhat contradictory; this may be due in part to local names for the various species and the resultant confusion in their identification, as pointed out by Schuchardt (1886-87), Díaz and Vivanco ('42), and others.

We have studied in the rat, the nutritive values and toxicities of a number of species of *Lathyrus*, including two which are commonly associated with the condition of lathyrism in man, *L. sativus* and *L. cicera*, and also including *L. odoratus*. The results of this last study, which confirm and extend the observations of the Wisconsin group (Geiger, Steenbock, and Parsons, '33), have been published in preliminary form. (Lewis and Esterer, '43).

In addition, with the cooperation of Mr. Roland McKee of the Bureau of Plant Industry of the United States Department of Agriculture and his associates, we have been able to study five other species which have been of some interest in various parts of the United States, chiefly because of their usefulness as forage crops. Many of the *Lathyrus* species can be grown under conditions which are difficult or unsatisfactory for the production of other forage crops. We wish to

express our sincere appreciation to Mr. McKee and his associates for this help, which has made it possible to extend this study beyond the scope which was originally projected.

EXPERIMENTAL

Young white rats, 50 to 80 gm in weight, in litter units, were paired as to sex and weight. One animal of each pair received the experimental (*Lathyrus*) diet ad libitum, while the food consumption of the paired litter mate fed the control (edible pea) diet was limited by the food intake of the mate fed the experimental diet. Daily records of food consumption were kept.

Finely ground meal (20 mesh sieve) was prepared from the *Lathyrus* peas and, for the control diets, from the edible split white peas of commerce (*Pisum sativum*, var. *arvense*). Since it is known that many legumes supply protein of inferior biological value, 10% of casein, a protein of high biological value, was incorporated into all the diets. The pea meal was added at a level of 50% to insure a high content of the potentially toxic legume. The protein content ($N \times 6.25$) of the diets fed varied from 21.55% (edible white pea) to 26.9% (*Lathyrus cicera*), of which 10% was derived from the casein. With *Lathyrus tingitanus*, which because of its toxicity was fed at the 25% level, the total dietary protein was 19.57%. It is not believed that the differences in protein content of the diets representing various *Lathyrus* species (22.55–26.95%) were significant, particularly since, as will be noted later, best growth was obtained with those containing *L. sativus*, which were lowest in protein content of the rations containing *Lathyrus*.

The mixed diets were constituted as follows: pea meal, 50%; casein², 10%; corn starch, 27%; sucrose, 5%; salt mixture (modified Osborne–Mendel), 4% corn oil, 2%; and cod liver oil, 2%. The dry ingredients of the diet were thoroughly mixed and stored, and the oils added to the mixture, to make

² Labco, vitamin-free.

the complete diets as needed. In addition, each rat received 200 mg of dried yeast and, in later experiments, 100 mg of powdered liver extract³, which were added to the diet to supply the B factors in addition to the vitamins made available in the dried yeast.

Sources of legumes

1. The edible split white peas used for the control diets were supplied by Mr. Floyd Trail of the Washburn-Wilson Seed Company, Moscow, Idaho.

2. *Lathyrus odoratus*. The sweet pea seed was made available through the generosity of Mr. Raymond H. Coulter of the Ferry-Morse Seed Company, Detroit.

3. *Lathyrus sativus*. The seed was obtained from India through the cooperation of Dr. B. Mukerji of the Biochemical Standardization Laboratory, Calcutta. The seed was submitted to Mr. Paul Russell and Mr. Roland McKee of the Bureau of Plant Industry who, after examination of the seed and of young plants grown in greenhouse experiments, reported that the material was one of the "small seeded dark strains of *L. sativus*;" the close resemblance of this seed to that of certain strains of *L. cicera* was, however, noted.

4. *Lathyrus cicera*. Two samples of seed, grown in plantings of 1942 and 1943 at College Station, Texas, were obtained from Mr. Karl F. Manke and Mr. P. R. Johnson.

5. *Lathyrus hirsutus*. Two samples of the seed of this species, grown during different seasons, were obtained from Mr. Roland McKee, who stated that "this is the only species that is being grown extensively in the United States. This species is quite widely spread in the states of Louisiana, Mississippi, Alabama and adjacent territories" and "has been harvested and sold as a pasture plant."

6. *Lathyrus tingitanus*. Two samples of seed, grown in different seasons by the Division of Forage Crops and Diseases at Corvallis, Oregon, were obtained through Mr. H. A. Schoth.

³ Lilly.

7 and 8. *Lathyrus aphaca* and *Lathyrus sphaericus*. Seeds of these species were secured through Mr. McKee.

9. *Lathyrus sylvestris Wagneri*. Three samples of seed, two grown in different seasons at the Western Agricultural Experiment Station at Puyallup, Washington, and one grown at Corvallis, Oregon, were obtained through Mr. J. W. Daniels, Mr. D. Dickson, and Mr. Schoth, respectively. A sample of the cured foliage ("hay") was also received from Mr. Schoth. This pea ("flat pea") has been recommended as a promising pasture crop on rough or logged-off land (Grunder and Dickson, '48).

DISCUSSION

Lathyrus odoratus

As shown in table 1, rats fed a diet which contained 50% of sweet pea meal uniformly developed lathyrism. The symptoms were, for the most part, similar to those reported by Geiger, Steenbock, and Parsons ('33) and included incontinence, lameness, paralysis of the rear limbs, paralysis of the front limbs with loss of control of the wrists, and spinal curvature. The spinal curvature was observed chiefly in the thoracic region and was usually both ventral and lateral. The sternum was deformed, frequently to such an extent that the volume of the thoracic cavity was strikingly diminished. We observed no instances of the hernias⁴ reported by Geiger, Steenbock, and Parsons ('33). It was striking that, despite the markedly toxic character of the diet, the animals in most cases continued to consume the diet readily, almost to the point of death. There was a marked variation in the period which elapsed from the onset of symptoms (initial, 2 to 7½ weeks; marked paralysis, 3 to 14 weeks) to death. All of our animals fed the sweet pea diet succumbed, but the period of feeding varied from 5 to 35 weeks. Most animals died within 7 to 10

⁴In very recent experiments still in progress, in a series of 6 animals, we have observed one hernia in which a testis was involved. No hernias were observed in any of the animals referred to in table 1, nor in the subsequent experiments in which other species of *Lathyrus* were fed.

weeks. None of the control rats showed any symptoms or abnormalities.

We used young rats in our experiments, as already indicated. However, we were able to produce the same clinical picture with older rats (up to 250 gm body weight). The spinal curvatures were, however, much less marked and the time when the symptoms appeared was much less constant than with the young rats.

TABLE 1¹

Increments in weight and clinical observations (paralysis) of young white rats fed sweet pea, modified sweet pea and edible pea (control) diets

GROUP	DIET	AVERAGE GAIN PER 100 GM FOOD	CLINICAL
		<i>gm</i>	
1.	Odoratus (21)	20.6	7,8,5,5,8,10,11,10,5,7, 5,4,5,5,6,7,3,4,13,14
2.	White pea (16)	24.7	no paralysis
3.	Odoratus + 25% casein (2)	16.6	4,6
4.	White pea + 25% casein (2)	21.4	no paralysis
5.	Odoratus, baked (5)	14.7	7,7,6,7,8
6.	Odoratus, baked + 12 mg ascorbic acid daily (5)	15.7	7,5,5,6,6
7.	Odoratus, autoclaved (4)	26.4	4,5,5,6
8.	White pea, autoclaved (4)	31.4	no paralysis
9.	Water-extracted odoratus + split pea extract ² (4)	20.2	no paralysis
10.	Water-extracted white pea + odoratus extract ² (4)	25.8	4,5,7,5
11.	40% white pea + 10% dried odoratus extract ³ (2)	22.7	11,11
12.	Alcohol (30%)— extracted odoratus (2)	17.8	no paralysis
13.	White pea + 1% phytin (5)	25.1	no paralysis
14.	White pea (5)	23.8	no paralysis
15.	Odoratus + liver extract (3)	27.4	9,7,7

¹ The gains per 100 gm of food are calculated for the first 4 weeks of the experimental period. The values in parentheses are the number of experimental animals in each group. The figures in the column headed "Clinical" represent the period in weeks at which symptoms of *marked* paralysis appeared. The diets all included 50% of pea meal and 10% of casein except where indicated (groups 3, 4 and 11).

² Aqueous extract.

³ 30% alcohol extract.

Through the cooperation of Dr. Fred J. Hodges and Dr. Robert W. Bryn of the Department of Roentgenology, it was possible to make a series of roentgenological studies at weekly intervals on a group of 6 rats fed the *Lathyrus* pea diet and a similar control group fed the white pea diet. We were thus able to observe the progressive changes in the skeletal structures as lathyrism developed (fig. 1). No path-

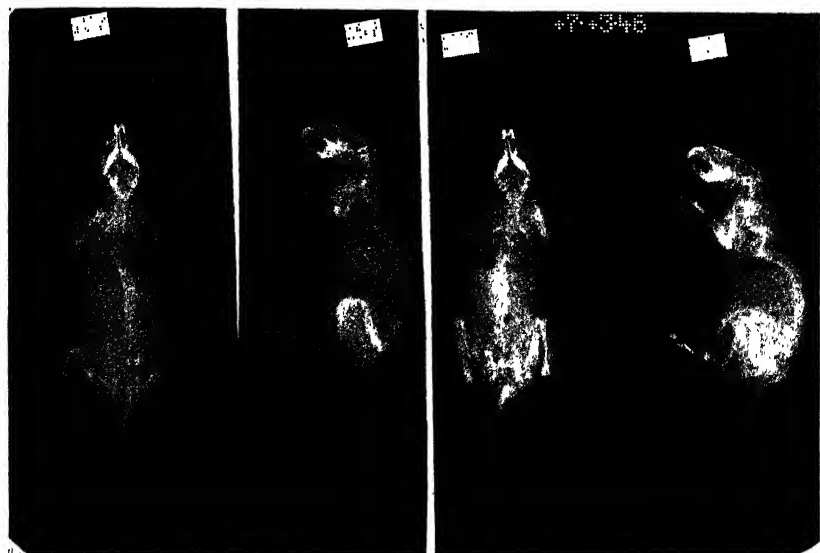


Fig. 1 Roentgenological observations on rat 157 fed the *Lathyrus odoratus* diet. The photographs at the left and right show the skeletal changes at 5 and 7 weeks respectively.

ological conditions were observed in the control group at any time.

The development of marked kyphosis and scoliosis of the spine with the apex of curvature in the mid- and lower-thoracic regions, observed in the skeletons at autopsy, was readily seen in the films. Curvature of the spine could be observed after two weeks on the sweet pea diet, and increased progressively during the period of observation. Although it would seem likely that there should be deformity in individ-

ual segments of the vertebrae due to compression, there appeared to be no gross evidence of such deformity or decalcification in the course of the development of the abnormal picture. The changes were postulated to be in the soft tissue-supporting structures of the spine, which were such that deformity could easily occur. The deformity of the bony thoracic cage was considered to be the result of the developed kyphosis of the spine.

Changes in the femur were similar to those seen in human osteomalacia. In the rats there occurred irregularities of the proximal third of the femur with thickening of the cortices, sub-periosteal calcification and multiple infractions near the end of the shaft. The changes in the tibia, for the most part, consisted of osteoperosis (demineralization). Most striking were the changes in the knee joints, with fragmentation of the epiphyses of the tibia, femur, and to a milder degree, of the patella. Marginal sclerosis was present and there was evidence suggestive of hemarthrosis (bleeding into the joint). The changes in the knee joints might be compared to those in Charcot's disease in man, which is a disorder of neurotrophic origin.

These *in vivo* observations were confirmed by autopsy. The changes in the spine, femur, and tibia were similar to those described by Geiger, Steenbock and Parsons ('33). The spinal column, bones of the hind limbs, and muscle were examined microscopically by Professor Carl V. Weller of the Department of Pathology, to whom we are indebted for the pathological reports. Characteristic findings on two animals are presented. In Rat 17, "the spine shows the same severe changes as are found in the long bones. There is a sharply localized collapse of the centra which must mean that there was an early decalcification. This has resulted in a compression of the spinal cord of so severe a degree that there is adequate explanation of the paralysis of the extremities. There is much new formation of connective tissue and cartilage and also periosteal hemorrhage. The proliferation of connective tissue extends into the neighboring muscle." In

Rat 5, the bone marrow, after decalcification, "is in part fatty myxomatous and there is much less hematopoiesis than in the control animal. There has been a new formation of bone upon the original cortex and a sclerotic layer has developed in the periosteum. Epiphyseal lines are widened, particularly at the upper end of the femur In addition to the effect in hematopoiesis, there is a delayed ossification (such as is seen in rickets), while new formation of bone around the shaft is more suggestive of a late stage of scurvy."

The toxicity of the *Lathyrus* pea diets was not altered when extra casein was added to give a total dietary protein content of approximately 38% (25% casein), the extra casein replacing an equivalent amount of starch in the diet. The presence of considerable amounts of good animal protein did not delay or prevent the onset of lathyrism (cf. Groups 3 and 4, table 1). The failure of casein in concentrations of either 10 or 25% in the diet to prevent the development of lathyrism is of interest, since it has been suggested that lack of adequate amounts of dietary tryptophane may be a factor in lathyrism (Díaz, '41). Certainly the tryptophane content of our casein-pea meal diets was such that there could have been no tryptophane deficiency.

The sweet pea meal was mixed thoroughly with an equal weight of water and the mixture was baked in an oven at 80° for 24 hours. When the baked meal was incorporated into the diets, there was no change in the toxicity (Group 5, table 1). More drastic heat treatment was obtained by autoclaving both the sweet pea and white pea meals at 120° for 6 hours. Diets in which the autoclaved sweet pea meal was present (Group 7, table 1) were no less toxic than those which contained the raw meal. It is therefore evident that, as noted by Geiger, Steenbock and Parsons ('33), the toxic principle of sweet peas is thermostable, even when autoclaved. There is considerable evidence that cooking frequently increases the biological value of otherwise inferior leguminous protein (Waterman and Johns, '21; Johnson, Parsons, and

Steenbock, '39; Lewis and Taylor, '47). It is of interest to note the somewhat better gains per 100 gm of food consumed which were observed when the autoclaved pea meals, both the sweet and white pea, were fed (cf. Groups 1 and 7 and Groups 2 and 8, table 1).

In a further study of the properties of the toxic agent present in sweet peas, the meal was extracted overnight at 0° with distilled water. The extract was decanted and the extraction repeated three times. The combined extracts were evaporated to a thick syrup *in vacuo*. A similar extraction of white pea meal was carried out. To the extracted white pea meal residue was added the concentrated extract of sweet peas, and to the extracted sweet pea meal residue was added the extract of white peas. The combined extracts and residues were then dried in thin layers at 80°, finely ground, and the mixtures incorporated into the diets. All of the animals receiving the *Lathyrus* extract-white pea meal residue diet (Group 10, table 1) showed the characteristic symptoms of acute lathyrism, while none of the group fed the white pea extract-sweet pea meal residue diet (Group 9, table 1) exhibited any signs of toxicity. It was thus shown that the toxic material was readily extracted by cold water from the sweet pea meal and that the residual extracted meal was non-toxic, a finding in accord with that of Geiger, Steenbock and Parsons ('33) who used boiling water in their experiments. The toxic agent could not be removed by extraction of the meal with cold ether or 95% alcohol. After a series of trials, it was found that the most efficient removal of the toxicant could be effected by slow percolation of the meal with 30% alcohol in the large percolators commonly used for the extraction of drugs in pharmaceutical procedures. When sweet pea meal thus extracted was incorporated into the diet (Group 12, table 1), no toxicity resulted. On the other hand, when the dried 30% alcohol extract of sweet pea meal was added to white pea meal and the mixture incorporated into the diet (Group 11, table 1), the toxicity was similar to that observed

when a control group of animals was fed the unextracted sweet pea meal diet.

It has been suggested (Stockman, '34a, '34b) that an important factor in lathyrism and other intoxications caused by eating the seeds of certain legumes may be the presence of a toxic organic phosphate, possibly phytin or phytic acid, and that lathyrism results when this ester fails to be split by the phosphatase in the alimentary canal and is absorbed without hydrolysis. Although in the light of more recent studies of phytin this hypothesis seemed improbable, we tested it by feeding edible white pea diets to which 1% of phytin⁵ had been added. Although the experiments were continued for a period longer than that required for the development of skeletal changes and other symptoms of lathyrism (14 weeks), no toxic effects were observed (Group 13, table 1), nor was the growth of the animals inferior to that of the control group fed the edible pea diet without added phytin (Group 14, table 1).

We have observed that the addition of liver extract to the diet of rats has frequently resulted in increased food consumption and improved growth. Powdered liver extract⁶ in amounts of 100 mg per day was added to the diet of the rats fed the sweet pea meal. These animals were paired with rats fed the sweet pea diet alone, whose food consumption determined the amounts of food offered the group fed the liver extract. The gains per 100 gm of food for the initial 4-week period were 24.9, 14.8 and 19.2 gm for the *odoratus* group as compared with 26.0, 29.4 and 26.6 for the liver extract group. Despite this superior gain, the addition of powdered liver extract failed to protect against the onset of lathyrism (Group 15, table 1).

Both the roentgenological examination of the living animals and the pathological examination of the tissues indicated the presence of hemorrhagic changes, suggestive of scurvy.

⁵ We wish to express our appreciation to the Staley Manufacturing Company for the gift of the phytin used in these experiments.

⁶ See footnote 3, page 540.

Although the rat is able to synthesize ascorbic acid and does not require its presence in the diet, it was thought possible that the toxicant might in some manner have increased the ascorbic acid requirement, so that the animal was unable to synthesize it in adequate amounts. A condition such as this was observed in mice by Wolley and Krampitz ('43). This species also does not require dietary ascorbic acid, being able to meet its needs by synthesis. If glucoascorbic acid, a homologue of ascorbic acid containing 7 rather than 6 carbon atoms, were fed, the mice developed severe scurvy, comparable to the experimental scurvy seen in guinea pigs. These observations were interpreted as indicating a biological competition between the glucoascorbic acid and the ascorbic acid synthesized by the animal. The addition of liberal amounts of ascorbic acid to the *Lathyrus* diet afforded no protection against the production of lathyrism. Rats fed the sweet pea diet with or without added ascorbic acid (12 mg daily) developed lathyrism of essentially the same severity and within the same period of time (Groups 5 and 6, table 1). The content of ascorbic acid in the plasma and tissues[†] of a group of control animals which were fed the stock diet, and of the groups which received the sweet pea diet with and without added ascorbic acid, was determined. No differences in the values were observed (table 2). The ascorbic acid was determined by the Bessey ('38) modification of the procedure of Mindlin and Butler. It would appear that a disturbance in ascorbic acid metabolism is not a factor in the causation of lathyrism by the sweet pea diet used in the present study.

Two theories of the cause of lathyrism are commonly proposed: lathyrism may be a "deficiency" disease or it may be occasioned by the presence of an intoxicant (presumably a "neurotoxin") in the seeds of the *Lathyrus* species. Deficiencies in protein and in vitamins have been suggested. The poor biological value of the proteins of many legumes is recognized and, in particular, claims have been made that *Lathy-*

[†] These determinations were carried out by Mrs. Naomi Levine Foa, to whom we are indebted.

rus pea protein is deficient in tryptophane. In the present experiments, in which casein at a level of 10% (or, in a few animals, 25%) was present in the diet, a deficiency in or a low biological value of the protein, and certainly a low content of dietary tryptophane, can hardly be the basis of the nutritive failure of the animals. Moreover, heat treatment of the sweet pea meal, a treatment which frequently increases the biological value of leguminous proteins, failed to influence the development of lathyrism.

TABLE 2¹

Ascorbic acid content of tissues of control rats and of rats fed the sweet pea diet with or without the addition of ascorbic acid

TISSUE	D I E T		
	Stock	<i>Lathyrus peas</i>	
		<i>No supplement</i>	<i>Ascorbic acid</i>
Liver	0.24 (0.15-0.32)	0.19 (0.15-0.27)	0.21 (0.16-0.25)
Kidneys	0.10 (0.06-0.13)	0.12 (0.09-0.14)	0.13 (0.06-0.16)
Adrenals	2.36 (0.91-4.2)	2.86 (2.2-3.4)	2.61 (1.9-3.1)
Plasma	0.47 (0.22-0.96)	-0.26 (0.14-0.38)	0.49 (0.35-0.61)

¹ Average values for three groups of 5 animals each are given with the ranges in parenthesis. All values are expressed as milligrams per gram of tissue or per 100 ml of plasma. The rats fed ascorbic acid received 12 mg per day.

Among vitamins, lack of which has been believed to be a factor in lathyrism, are vitamin A and the B complex group. In all our experiments, the rats received vitamins A and D in the form of cod liver oil. None of our control animals exhibited the symptoms of lathyrism as did the animals fed the sweet pea diet, as might be expected if deficient fat-soluble vitamins were related to lathyrism. Water-soluble vitamins were supplied by dried yeast powder. The onset of lathyrism was not delayed in our animals by the addition to

the diet of liberal amounts of liver extract. This observation is opposed to the findings of Díaz and Vivanco ('42) and Díaz, Vivanco and Mendoza ('43), who were able to prevent the development of toxicity in rats⁸ fed *Lathyrus cicera* by feeding crude liver extract. Since their autoclaved extract failed to protect the animals, the presence of a new water-soluble thermostable protective factor in liver was postulated.

The results of our experiments with *Lathyrus odoratus* suggest that lathyrism is related to a thermostable toxic agent, which cannot be extracted from the seed by ether but which is removed readily by cold water or by 30% alcohol. Experiments with other species of *Lathyrus* support this observation.

The growth of the young animals fed other species of *Lathyrus*, which were not acutely toxic, is shown in table 3. The growth responses calculated per 100 gm of food for periods of 4 and 7 weeks respectively are presented for both the experimental groups (fed the *Lathyrus* peas) and their paired controls (fed the edible white pea). Since, in certain cases, definite failures in food consumption were observed and toxicity developed as the experiment progressed beyond 4 weeks, the gains in the 1st 4 weeks are perhaps more significant. The number of animals is the number of rats fed the experimental diets; the number of control rats (edible split pea diet) equalled them in all cases. The experiments with certain species were continued beyond 7 weeks; this is indicated in the column "Total period." The last column ("Clinical") presents the time required to develop symptoms of paralysis, lameness and similar indications of experimental lathyrism.

Lathyrus sativus

Contrary to observations in both man and many domestic animals, in whom lathyrism, both experimental and clinical, has been noted after the feeding of this species, we have ob-

⁸ Díaz and his co-workers observed toxicity in rats fed *Lathyrus cicera*, but emphasized that the symptoms were not those of lathyrism as seen in either man or the horse.

TABLE 3¹
Growth of young white rats fed various species of Lathyrus

LATHYRUS SPECIES	NUMBER OF RATS	GAINS PER 100 GM FOOD						CLINICAL NOTES
		4 weeks		7 weeks		Total period		
		Experimental group	Control group	Experimental group	Control group			
		gm	gm	gm	gm	wks.		
Sativus	6	21.6	23.9	19.4	21.2	14	None	
Sativus	4	28.8	28.2	21.9	19.8	7	None	
Cicera	6	22.1	22.4	20.8	20.1	7	None	
Cicera (new lot)	3	19.2	22.3	15.9	17.1	21	None	
Aphaea	5	14.9	30.4	18.8	30.4	17	Non-typical	
Hirsutus	4	23.1	...	17.9	...	27-43	25, 27, —, 28 *	
Hirsutus (new lot)	3	19.2	26.8	14.4	24.1	7-23	11, 10, 6	
Tingitanus *	5	-2.5	6.8	3.6	7.5	12-33	25, 25, 12, 25, 33	
Tingitanus (extracted)	3	25.7	25.1	14.6	17.8	12-14	None	

¹ All the animals received diets containing 50% pea meal and 10% casein except the *L. tingitanus* group, to which 25% pea meal was fed. The figures in the column headed "Clinical notes" represent the period in weeks in which symptoms of marked paralysis appeared. The animals fed *L. tingitanus* showed the osseous malformations associated with lathyrism, but developed no marked paralysis.

² Died in 43 weeks.

³ All animals of this group were either dead or killed at the period (weeks) indicated in the last column.

served satisfactory growth in rats fed this legume over periods of 7 to 14 weeks. In the first series of 6 rats (table 3), the food intake of the experimental group was limited to 10 to 12 gm daily, while in the 2nd series the voluntary food intake often reached 16 to 17 gm daily. The animals ate the *L. sativus* more eagerly than any other experimental or control diet. The gains per 100 gm of diet were essentially the same as those of the litter mates fed the white pea diet.

These results are in agreement with those of Díaz and Vivanco ('42), McCarrison and Krishman ('34), Visco ('24b) and Zagami ('31), who were unable to observe symptoms of lathyrism in young rats fed *L. sativus*. McCarrison and Krishman ('34), however, who fed *L. sativus* as the sole component of the diet, reported that the animals refused food after 6 weeks and died from asthenia. The animals of Díaz and Vivanco ('42), which received diets composed exclusively of raw or cooked *L. sativus*, also grew less rapidly than the controls on a mixed diet, although no deaths or signs of lathyrism were observed in 8 or 9 weeks. Zagami ('31) also observed retarded growth and failure of normal development, even though symptoms of lathyrism were not present. Our animals fed *sativus* at the 50% level made good gains and continued to grow throughout a period of 14 weeks.

Lathyrus cicera

The ingestion of this species of *Lathyrus* by both man and domestic animals (Schuchardt, 1886-87; Stockman, '29) has led to the development of lathyrism, although it has not been possible to produce lathyrism by feeding *L. cicera* to rats, even when the diet is composed exclusively of this pea. Similar results with rats were obtained in our own experiments in which *L. cicera* was fed at the 50% level. The rats ate the diet well; good growth comparable to that of control animals fed the edible split pea of commerce was obtained; and no symptoms of lathyrism were noted (table 3). No spinal curvature or abnormalities of the long bones were seen at autopsy.

Lathyrus aphaca

None of the rats fed diets containing this pea developed any of the characteristic symptoms of lathyrism within a period of 17 weeks. Although no acute toxicity was apparent, the gains per 100 gm of food eaten were definitely less than those of the pair-fed control group which received the edible white pea diets (table 3). There appeared in several of the animals some alopecia, notably around the neck. The addition of inositol to the diet failed to influence the development of the alopecia in these animals. It is evident that the acute toxic factor associated with *L. odoratus* and some other species of *Lathyrus* to be discussed is not present in *L. aphaca*, but that the seed, presumably the protein of the seed, is inferior from the nutritive standpoint to *L. sativus*, *L. cicera*, or *Pisum sativum*.

Lathyrus hirsutus

In the 1st series of experiments with this species of *Lathyrus*, the animals grew moderately well for 13 or 14 weeks and up to that time showed no signs of paralysis or spinal curvature. Subsequently, however, they lost weight, developed paralysis and spinal curvature and died in 26 to 43 weeks. We have never observed so long a period of survival in rats fed a diet containing the sweet pea (*L. odoratus*). On the latter diet the rats developed symptoms of paralysis in 7 weeks or earlier and in no cases survived more than 16 weeks. We were able to secure a 2nd sample of *L. hirsutus* seeds grown in a different season, but in the same region. On a diet containing these peas, growth was poorer and the animals developed the characteristic symptoms of lathyrism in 6 to 11 weeks. It is evident that the seeds of *L. hirsutus* are toxic and produce symptoms similar to those occasioned by *L. odoratus*, although the toxicity is definitely less. Pathological changes in the long bones and spinal column similar to those produced by diets containing *L. odoratus* were reported by Dr. Carl V. Weller, to whom we are indebted for the pathological studies.

Lathyrus tingitanus

This seed, when fed at a level of 50% in the diet, proved very toxic. The animals ate the diet poorly and died in from 8 to 25 days. Thus rat 83 consumed only 20.5 gm of the mixed diet (equivalent to approximately 10 gm of the pea meal) over a period of 9 days and died on the 9th day. The addition of 100 mg of powdered liver extract⁹ to the diet, while it resulted in some increase in food consumption, did not in any way decrease the toxicity. When *L. tingitanus* was incorporated into the diet at the lower level of 25%, the animals survived for longer periods of time but lost weight in the 4-week period and had made little recovery at the end of the 7-week period (table 3). Of a group of 5 animals fed at this level three died and two were killed at the end of 25 and 33 weeks respectively. All of the animals of the group developed spinal curvature and the other skeletal changes characteristic of lathyrism in the rat. Addition of powdered liver extract over a period of 15 weeks failed to improve the growth or to prevent the development of symptoms. When the rats were fed the seed extracted with 30% alcohol as described previously, the diet was eaten readily even at the 50% level and the animals made essentially the same gains as the controls (table 3). No signs of lathyrism were observed in 12 to 14 weeks. This is in marked contrast to the experiments with the unextracted seed in which, at the 50% level, the food consumption was poor, the animals lost weight rapidly, and died in from 8 to 25 days. It is evident that *Lathyrus tingitanus* contains a toxic substance, extractable by 30% alcohol, and that the extracted pea meal is of good biological quality as evidenced by gains per 100 gm of food consumed.

*Lathyrus sphaericus*¹⁰

This seed also proved to be highly toxic when fed at a level of 50% of the diet. The rats ate the diet poorly even when

⁹ See footnote 3, page 540.

¹⁰ These experiments were carried out with the cooperation of Prahlad C. Rajam.

powdered liver extract was added and young animals of 40 to 50 gm body weight died in 4 to 8 days. With slightly older and larger animals (85-95 gm), the period of survival (12, 14, 23 and 38 days for one group) was longer, but the symptoms were similar. Even with fully grown rats of 275 to 300 gm in weight, death resulted in 25 to 45 days. The animals lost weight rapidly; this was in part due to low food consumption, but it should be pointed out that the loss in weight in the paired controls was only about 50% of that of the experimental groups. The animals became weak and gradually lost control of their movements; the hind legs became stiff and later paralyzed. Since the young animals died after so short a period on the experimental diet, it was hardly to be anticipated that the skeletal changes seen in animals fed the seeds of *L. odoratus* or *hirsutus* would be observed.

Two rats of 115 to 125 gm body weight were fed the meal of *L. sphaericus*, previously extracted with 30% alcohol. The animals ate well and developed no symptoms of toxicity over a period of 21 days. Unfortunately, our limited supply of seed prevented further studies along this line.

Lathyrus sylvestris Wagneri

Of all the species of *Lathyrus* studied, this pea was the most toxic. When fed at the 50% level, the animals refused food within two to three days and died within 4 to 10 days. In many cases, symptoms of intoxication developed after the animal had eaten as little as 4 gm of the legume (8 gm of the mixed diet). The symptoms of intoxication were striking; the animals became hyperexcitable and during convulsive seizures would stand upright with head thrown back and with increased and spasmodic respiration. These seizures were repeated at frequent intervals and could be induced readily by external stimuli (touching animal with a rod, knocking on the cage, etc.). We observed the same symptoms of intoxication when seed grown in both Washington and Oregon and in different seasons was fed. With larger rats, the same picture was presented but the symptoms were somewhat delayed.

One rat of 250 gm survived the acute stages of toxicity and was fed the laboratory stock diet for over 8 months. However, it was possible to induce convulsive seizures by external stimuli even up to approximately 8 months after the diet containing *L. sylvestris* was withdrawn.

The same results were obtained when a diet containing 50% of finely pulverized *sylvestris* hay and 20% of casein, or the hay alone, was fed to larger rats. Characteristic symptoms and death resulted in from 6 to 13 days. In one experiment, the animal had consumed only 4 gm of hay prior to death.

The toxic agent could not be extracted from the pea meal by ether but could be removed by extraction with 30% alcohol. Gains of 11.7, 15.0 and 23.2 gm per 100 gm of food were obtained with the extracted pea meal, gains comparable to those of control animals under similar conditions of diet but receiving white peas.

The results of our studies with 8 species of *Lathyrus* have shown the presence of a factor in the seeds, with the exception of those of *L. sativus*, *L. cicera* and *L. aphaca*, which is toxic for rats under the conditions of our experiments. It is yet to be determined whether experiments carried out for longer periods of time would reveal the presence of a toxin for rats in the three apparently nontoxic species. It is notable that two of these species (*L. sativus* and *cicera*) have been commonly associated with human lathyrism. In those species in which we have undertaken to extract the intoxicant, the principle has been soluble in 30% alcohol and the residual meal after the alcohol extraction has been devoid of toxicity and as effective in the promotion of growth as the white split pea of commerce, when fed at the 50% level with 10% of casein.

Of the species studied, *Lathyrus sylvestris Wagneri* has proved most toxic. Its action suggests the presence of a very powerful neurotoxin. It is of interest to note here the careful description of the pathological anatomy of a human case of lathyrism (*Lathyrus sativus*) reported by Filimonoff

('26), in which extensive injury to the nervous system was observed. He also mentions many observations from the older Russian literature not available to us. Of particular significance is the citation (p. 79) of the experiments of Spirtoff with dogs in 1903, in which acute lathyrism with marked nervous injury was produced by prolonged feedings of *L. sativus*, *clymenum*, *cicera* and *sylvestris* in considerable amounts. Of these, *Lathyrus sylvestris* was most rapidly toxic and produced more severe injury to the gray matter of the cerebrum, notably the cortex, than did the other peas. The toxicity of the foliage of this species for certain domestic animals is recognized in our Western states (Grunder and Dickson, '48; Haag, '48). Further studies of the nutritive value of *Lathyrus* species are in progress in this laboratory.

SUMMARY

The nutritive values of the seeds of a number of species of *Lathyrus* of importance in human or animal nutrition when fed at a level of 50% with 10% of a biologically superior protein (casein) and with adequate minerals and vitamins have been studied in young white rats.

In confirmation of the studies of Geiger, Steenbock and Parsons ('33), nutritive failure and experimental lathyrism resulted when young white rats were fed diets containing ground sweet peas (*Lathyrus odoratus*). Since the diets contained also 10% (in a few experiments, 25%) of casein, a protein or amino acid deficiency is not believed to be a causative factor.

The condition is believed to be associated with the presence of an intoxicant, which is stable to heat (24 hours at 80°, 6 hours at 120° in an autoclave) and which can be extracted readily by cold water or 30% alcohol. The extracted sweet pea meal was not toxic.

Although roentgenological and pathological observations indicated certain changes suggestive of scurvy, the addition of 12 mg of ascorbic acid daily to the diet failed to prevent lathyrism. The ascorbic acid content of the blood and tissues

of rats fed the sweet pea diet and the sweet pea diet plus ascorbic acid was the same as that of control animals of the same age fed the usual stock diet. When liver extract was added to the sweet pea diet, the development of lathyrism was neither prevented nor delayed.

When phytin was added to our control (white pea) diets, no toxicity was observed.

Lathyrus sativus and *cicera* showed no toxicity and promoted growth as effectively as did the edible white split pea of commerce. *Lathyrus aphaca* was nontoxic, but was inferior in growth promotion to the other species.

Lathyrus hirsutus, *tingitanus*, *sphaericus*, and *sylvestris Wagneri* were toxic, and the last three were unacceptable to the rats (they are listed in order of increasing toxicity). Of these, *Lathyrus sylvestris Wagneri* was most acutely toxic and appeared to contain a toxic substance which produced marked injury to the nervous system. The toxic material of *L. tingitanus*, *sphaericus* and *sylvestris Wagneri* could be extracted by 30% alcohol and the extracted meal was effective in promoting growth.

With the species less acutely toxic (*L. hirsutus* and *tingitanus*), it was possible to obtain the skeletal changes described as typical of lathyrism.

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STUDIES ON BERIBERI IN AN ENDEMIC SUB-TROPICAL AREA ¹

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Beriberi ranks very high in the Philippines as a cause of illness and of death. During 1946 and 1947 it ranked second only to pulmonary tuberculosis as a cause of death. The respective mortality rates per 100,000 population for the two diseases were 148.21 and 170.06 in 1946. In 1947 beriberi claimed 24,296 lives with a mortality rate of 131.92 per 100,000, compared with 116.13 in 1939 and 111.87 in 1940. About two-thirds of the deaths are of infants.

While there are still many unknown factors in the etiology of beriberi, it has been commonly accepted that in rice-eating countries the disease is due, in a practical sense, to the consumption of large quantities of highly polished rice. Clinical beriberi is endemic in certain areas in the Philippines where rice is the chief staple food. Mortality from beriberi tends to be highest in those provinces where rice is grown most intensively.

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An attempt is projected to reduce the incidence of beriberi in an endemic area in the Philippines by trial of artificial enrichment of the entire rice supply. Bataan was selected because of its high beriberi mortality, the presence of only one national highway, whereon rice traffic may be controlled readily, and the generally uniform economic and agricultural conditions in all parts of the province. This paper deals with the findings of an 8-month clinical beriberi survey in that province, in contemplation of the introduction of enriched rice later in 1948. For comparison the survey will be repeated at a later date.

Metcoff and his co-workers ('45) described a nutritional survey at Norris Point, Newfoundland, in June 1944. The survey was done with a two-fold purpose; (1) rapid assessment of nutritional status; (2) establishment of a baseline for comparison with conditions after the then-contemplated introduction into Newfoundland of enriched flour. Our study thus has a pattern similar to that of Metcoff et al.

Of the 12 towns in Bataan, 9 were surveyed by sampling. From 11.11% to 64.04% (average 22.26%) of the total population in each town was examined clinically. The total number of persons examined was 12,384, among whom 1,580 cases of frank or suspected beriberi were encountered. The cases of beriberi were classified as frank or apparent, suggestive, and clinically doubtful. The population surveyed was classified as (a) infants; (b) children; (c) expectant mothers; (d) nursing mothers; (e) other adults.

MATERIALS AND METHODS

Three survey teams, each consisting of a full-time Medical Officer and a Clerk-Helper, were constituted and three municipalities were assigned to each team. Each physician made a thorough physical examination of 1,000 to 2,500 random subjects in each municipality assigned to him. Municipalities in the Philippines consist of a central town (*poblacion*) usually surrounded by several villages (*barrios*). With few exceptions, the survey included a proportionate sample from

the central town and from each barrio. Two of 12 barrios in Hermosa were omitted because of disturbed socio-economic conditions there.

The physical examination included the taking of blood pressure, height and weight, and an examination for beriberi symptoms as indicated in table 3. The age, sex, occupation, address, family income per month, number of members of the family, other dependents, and similar pertinent data were recorded. Subjects who showed clinical beriberi, including clinically doubtful or suspicious cases, were referred to physicians on part-time service with the local units of the Bureau of Health. Clinical diagnosis of beriberi was accepted only when confirmed by the latter group of physicians.

Metcoff et al. ('45) classified nutritional deficiencies thus: (1) apparent, when the subject exhibited three signs or two signs and one or more symptoms characteristic of a dietary deficiency; (2) suggestive deficiency, when the subject had two signs or one sign and one or more symptoms; and (3) questionable deficiency if only one sign was present or if signs were less definite. In the present survey, the classification adopted by Metcoff was followed using the terms *frank or apparent beriberi*, *suggestive beriberi* and *clinically doubtful beriberi*.

Dann and Darby ('45) in discussing the zones of nutriture defined 5 zones or levels as follows: (1) saturated; (2) unsaturated, but functionally unimpaired; (3) potential deficiency disease; (4) latent deficiency disease, which was described by them as the mildest clinically-detectable form of deficiency characterized by "vague, indefinite, non-specific symptoms," and (5) clinically manifest deficiency disease which is divisible into mild or severe forms. Mild deficiency is that stage of the disease in which the classical syndrome has not yet been manifested but there are present highly suggestive signs and symptoms sometimes requiring laboratory confirmation and therapeutic tests to establish the diagnosis. The classical syndrome is present in severe deficiency. In this work, clinically doubtful beriberi falls under the classi-

fication of latent deficiency, and suggestive and frank beriberi are under the categories of mild and clinically manifest severe deficiency, respectively. Continuing, these authors re-stated the concept of the progressive development of deficiency diseases from a diminished concentration of the vitamins in the blood, succeeded by decreasing body stores, leading subsequently to a functional impairment, then to microscopic and finally to gross anatomic changes. In assessing nutriture they listed clinical examinations, demonstrations of a biochemical or physiological lesion, and estimates of the past dietary intake as the methods used. They emphasized, however, that estimation of the dietary intake is of secondary value at present, especially in potential or latent deficiencies. Similarly, Gordon ('47) divides the development of deficiency diseases into 4 stages: (1) suboptimal intake of one or several nutritional factors; (2) depletion of storage reserves; (3) appearance of a biochemical lesion detectable by chemical tests; and (4) development of tissue changes.

Darby and Milam ('45) in a field study of the prevalence of clinical manifestations of dietary inadequacy included in their survey a medical history, a physical examination, a series of laboratory determinations of blood specimens, and the recording of individual 7-day food intakes from which the average daily intake of the various nutrients was determined by the use of food tables. King ('48) noted that chemical methods of appraising nutritional status are being more widely used in order to furnish a better basis for diagnosing deficiencies, not by replacing but by supplementing dietary histories and clinical examinations.

In this survey the signs and symptoms of clinical beriberi were listed on a card on which the examining physician indicated for each person the presence or absence and the severity of existing signs and symptoms (see table 3). In drawing up the card of symptoms and signs, the descriptions by Jolliffe ('45), Albert and Abad ('47), Eddy and Dalldorf ('41), Youmans ('43), McLester ('43), Wohl ('45) and Hibbs ('46) were consulted.

FINDINGS AND OBSERVATIONS

The incidence of clinical beriberi in Bataan, in people of various ages and physiological conditions, is presented in tables 1 and 2, the findings being classified by degree of deficiency and place of residence. An analysis of the data

TABLE 1

Incidence of clinical beriberi in Bataan by age, physiological condition and degree of deficiency

POPULATION GROUP	FRANK BERIBERI			SUGGESTIVE BERIBERI			DOUBTFUL BERIBERI			TOTAL		
	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)
	no.	%	%	no.	%	%	no.	%	%	no.	%	%
Infants (0-2 years)	5	0.48	.040	12	1.07	.097	16	1.56	.129	33	3.03	0.27
Children (2.01-15 years)	13	0.43	.105	7	0.28	.057	24	0.68	.194	44	1.58	0.356
Expectant mothers	43	6.65	.346	74	11.45	.597	122	18.88	.985	239	36.99	1.93
Nursing mothers	79	4.78	.636	120	7.27	.970	291	17.63	2.350	490	29.69	3.96
Other adults	105	1.82	.845	230	4.16	1.860	439	7.60	3.540	774	13.41	6.24
Total population	245	...	1.99	443	...	3.58	892	.	7.24	1580	...	12.76

(a) Number of cases found.

(b) Per cent incidence in group.

(c) Per cent incidence in population.

shows the following: (a) There is no significant difference in the incidence of clinical beriberi between the urban population ("poblacion") and the rural population ("barrio"); (b) among the groups specified in table 1, the incidence of clinical beriberi, with reference to the number of persons examined in each group, is highest in "expectant mothers," then in "nurs-

TABLE 2

Incidence of clinical beriberi in Bataan by degree of deficiency and place of residence

MUNICI- PALITY	"POBLACION" (CENTER) OR "BARRIO" (VILLAGE)	FRANK BERIBERI		SUGGESTIVE BERIBERI		DOUBTFUL BERIBERI		TOTAL	
		Cases (a)	Incidence (b)	Cases (a)	Incidence (b)	Cases (a)	Incidence (b)	Cases (a)	Incidence (b)
		no.	%	no.	%	no.	%	no.	%
Hermosa (26.95%)	Poblacion (22.4%)	9	1.69	17	3.19	57	10.71	83	15.60
	Barrios (31.24%)	6	0.78	15	1.95	68	8.94	89	11.58
	Total	15	1.15	32	2.46	125	9.61	172	13.23
Orani (15.88%)	Poblacion (11.39%)	2	1.88	1	.94	9	2.54	12	11.32
	Barrios (16.31%)	14	.87	25	1.56	109	6.83	148	9.28
	Total	16	.94	26	1.53	118	6.94	160	9.41
Samal (15.42%)	Poblacion (12.73%)	3	.33	14	1.56	65	7.29	82	9.21
	Barrios (20.86%)	2	.27	11	1.52	53	7.36	66	9.16
	Total	5	0.37	25	1.55	118	7.32	148	9.18
Abucay (25.02%)	Poblacion (25.16%)	16	1.26	44	3.49	80	6.32	140	11.07
	Barrios (24.32%)	7	2.91	10	4.16	13	5.41	30	12.53
	Total	23	1.53	54	3.59	93	6.19	170	11.30
Balanga (11.11%)	Poblacion (0%)
	Barrios (11.11%)	24	1.52	121	7.93	225	14.75	370	24.26
	Total	24	1.52	121	7.93	225	14.75	370	24.26
Pilar (41.18%)	Poblacion (88.16%)	9	2.27	12	3.03	22	5.56	43	10.88
	Barrios (34.56%)	24	2.31	48	4.78	43	4.19	115	10.75
	Total	33	2.18	60	3.96	65	4.55	158	10.69
Orion (17.10%)	Poblacion (13.78%)	34	3.95	42	4.88	31	3.60	107	12.44
	Barrios (25.0%)	56	8.84	54	8.23	25	3.81	135	20.17
	Total	90	5.92	96	6.34	56	3.70	242	15.96
Bagac (64.04%)	Poblacion (64.04%)	37	2.86	19	1.47	18	1.39	74	5.72
	Barrios (0%)
	Total	37	2.86	19	1.47	18	1.39	74	5.72
Moron (61.37%)	Poblacion (66.03%)	2	.21	9	.95	51	5.38	62	6.54
	Barrios (44.09%)	0	0.00	1	.44	23	10.13	24	10.57
	Total	2	.16	10	.81	74	5.92	86	6.73
Total (9 out of 12 towns) (22.26%)	All Poblaciones (21.13%)	112	2.26	158	3.19	333	6.72	603	12.17
	All Barrios (23.50%)	133	1.67	285	3.54	559	7.03	977	12.25
	Total Bataan	245	1.99	443	3.58	892	7.34	1580	12.76

(Numbers enclosed by parentheses indicate % of population examined.)

(a) Number of cases found in area.

(b) Per cent incidence in population of area.

ing mothers," "other adults," "infants," and "children"; (c) however, in relation to the total population of the community, the highest incidence of beriberi is in "other adults," followed by "nursing mothers," then by "expectant mothers," "children" and "infants" (the low incidence in infants compared with the high mortality is a reflection of the rapidity of fatal terminations in such cases); (d) the incidence of the various degrees of beriberi follows a uniform pattern of distribution among groups; (e) considering the total population surveyed by the criteria of dietary deficiencies given by Dann and Darby ('45) and by Gordon ('47), the incidence of clinical beriberi in all population groups totals 1.99% for frank beriberi, 3.58% for suggestive beriberi and 7.24% for doubtful beriberi, or 12.76% for all types; (f) analysis of the raw data demonstrates no influence of sex outside of the physiological conditions of pregnancy and lactation; (g) the incidence by towns or municipalities varies from 5.72% of the total population surveyed in Bagac to 24.26% in Balanga, with an average of 12.76% for the total population of Bataan. Frank beriberi is lowest in incidence at Moron with 0.16% and highest at Orion with 5.92%, with an average of 1.99%. Suggestive beriberi varies from 0.81% in Moron to 7.93% in Balanga, with an average of 3.58%. Clinically doubtful beriberi had an incidence ranging from 1.39% in Bagac to 14.75% in Balanga, with an average of 7.24%.

Frequency of signs and symptoms in clinical beriberi

The frequency of signs and symptoms by age, physiological condition, and degree of beriberi is shown in table 3. A separate column of additional symptoms has been provided for infantile beriberi. As is noted in the table, the most frequent symptoms in infants are aphonia, cyanosis, opisthotonus, and purposeless motions. In children, the most frequent signs and symptoms are referable to the nervous system; next, to the skin, muscles and cardio-vascular system. In expectant and nursing mothers the majority of the signs and symptoms

TABLE 3

Frequency of signs and symptoms in clinical beriberi in Bataan by age, physiological condition and degree of deficiency¹

BODY REGION OR SYSTEM INVOLVED WITH SIGNS AND SYMPTOMS	INFANTS (0-2 YRS.) (33) ²			CHILDREN (2.01-15 YRS.) (44)			EXPECTANT MOTHERS (239)			NURSING MOTHERS (490)			OTHER ADULTS 15 YEARS AND OVER (774)		
	F	S	D	F	S	D	F	S	D	F	S	D	F	S	D
<i>Mouth:</i>															
Cyanosis	4	9		2			5	3		28	17		38	29	1
Numbness of lips							18	15	16	31	46	63	33	58	88
Tremor of tongue							1	1					5		
<i>Gastrointestinal:</i>															
Anorexia					1			6	2	6	9	10	7	12	27
Vomiting		2					2	1		9	2	1	6	2	
Constipation	1	1			1		4	8	11	8	21	30	17	22	49
Waist tightness				1			10	8	15	27	25	34	32	46	59
<i>Cardiovascular:</i>															
Fatigability				9	3	5	26	41	48	43	52	80	62	102	165
Palpitation				3		4	20	27	32	35	52	62	45	75	149
Tachycardia							12	4		9	5		14	21	1
Murmurs				1			2	1		4	2			1	
Enlarged heart				5	2		4	1		3			5	2	
Edema				1			21	32		7	11		20	33	1
<i>Nervous system:</i>															
Tingling				6	5	14	33	59	88	61	101	222	80	192	351
Numbness				14	7	18	37	58	97	66	113	254	89	203	398
Reflex changes				8	2		11	8		43	29		56	72	2
Paresthesia									6	6	5	11	7	12	26
<i>Skin and muscles:</i>															
Pallor		1		11	1		26	11		59	42		70	54	1
Tenderness (calf)				6	3	11	24	28	58	30	44	141	40	77	283
Cramps				8	6	11	38	56	93	60	68	175	76	151	216
<i>Infantile beriberi:</i>															
Ptosis															
Purposeless motions	3														
Opisthotonus	5														
Head drop	1														
Aphonia	2	10	16												

¹F = frank beriberi; S = suggestive beriberi; D = clinically doubtful beriberi.

²Number of cases examined.

are referable to the cardio-vascular system, the nervous system, skin and muscles. In other adults, the signs and symptoms are more or less uniformly distributed over all the organ systems involved.

*Deaths and mortality rates from beriberi in
Bataan Province*

To help in forming a baseline for future assessment of the influence of artificial enrichment of rice on beriberi mortality in Bataan Province, the deaths and mortality rates for this disease for the last semester and for the full year of 1947 are presented in table 4. Almost two-thirds of reported deaths from beriberi in the province of Bataan in the year 1947 occurred in the second semester coinciding with our survey. This is perhaps due in part to the fact that the people of Bataan and the Medical Officers of the Bureau of Health in that province became more aware of the manifestations of beriberi after the survey was started in that province. It is noteworthy that as is indicated in table 4 the greatest proportion of deaths occurred in infants. Infantile death rates were 140.1 per 100,000 population for the full year of 1947 and 91.4 per 100,000 population for the second semester, compared to total beriberi death rates of 166.5 and 107.7, respectively. No deaths of expectant mothers from beriberi were reported in the year 1947 and there were only 4 deaths of nursing mothers, three of which occurred in the second semester.

For purposes of comparison, the 1947 annual mortality rate for beriberi for the whole Philippines was 131.68 per 100,000 population and for the City of Manila, 83.75. The authors are of the opinion that official morbidity statistics are not complete in view of the frequent failure of hospitals and of private physicians to report cases of beriberi to the health authorities.

"Fasting hour" urinary thiamine analysis

Hou ('39) reviewed the literature describing several studies of the 24-hour excretion of thiamine in normal subjects. He

TABLE 4

Mortality from beriberi in Bataan, 1947¹ (figures in parentheses record deaths reported for period July 1 - December 31, 1947; other figures are for full year)

MUNICIPALITY	ESTIMATED POPULATION	INFANTS 0-2 DEATHS	CHILDREN 2.01-15 DEATHS	EXPECTANT MOTHERS DEATHS	NURSING MOTHERS DEATHS	OTHER ADULTS DEATHS	TOTAL POPULATION	
							Deaths	Rate per 100,000
Abucay	9,552	14 (7)	1 (1)	0	2 (2)	1 (1)	18 (11)	167.5
Bagae	2,226	1 (1)	0	0	0	0	1 (1)	44.9
Balañga	14,349	31 (19)	2 (2)	0	1	2 (2)	36 (23)	250.8
Dinalupihan	13,591	14 (8)	1 (1)	0	0	1	16 (9)	117.7
Hermosa	9,207	29 (18)	4 (3)	0	0	2 (1)	35 (22)	380.1
Limay	4,906	2 (1)	0	0	0	0	2 (1)	40.7
Mariveles	5,491	4 (3)	0	0	1 (1)	0	5 (4)	91.05
Moron	3,107	7 (6)	0	0	0	0	7 (6)	225.2
Orani	11,739	12 (12)	1 (1)	0	0	1	14 (13)	119.2
Orion	10,372	12 (11)	0	0	0	6 (1)	18 (12)	173.5
Pilar	6,251	5 (2)	0	0	0	0	5 (2)	79.9
Samal	7,666	7 (2)	0	0	0	0	7 (2)	91.3
Total Bataan	98,457	138 (90)	9 (8)	0	4 (3)	13 (5)	164 (106)	166.5

¹ Data furnished by District Health Officer of Bataan.

and the authors he cited believe that thiamine excretion in the urine is dependent upon dietary intake. The figures for daily excretions in 24 hours range from 70 to 510 μg in normal subjects. In beriberi cases excretion may fall to zero. Some data published in 1939 showed 24-hour excretions in normal subjects ranging from 60 to 895 μg . Holt and Najjar ('43) believe that a 12-hour overnight fast should precede the collection for thiamine analysis of urine accumulated in the bladder during the 13th hour only. Hence the term "fasting hour excretion test." Johnson, Henderson, Robinson and Consolazio ('45) studied the comparative merits in field nutritional surveys of fasting specimens, random specimens and oral loading tests. They found that in 46 normal subjects with no previous load, the fasting hour excretion is 6 to 24 μg of thiamine, with a mean of 11 μg . They are of the opinion that fasting levels are more sensitive measures when other tests cannot be done and that the fasting rates of urinary excretion are more sensitive measures of vitamin intake in the previous three weeks than the 4-hour oral load tests. In this survey the "fasting hour" urinary excretion of thiamine after a 12-hour overnight fast was determined by the method proposed by Mickelsen, Condiff and Keys ('45). The results of the "fasting hour" urinary thiamine excretion test of beriberi cases in Bataan broken down with respect to age, physiological condition and degree of beriberi are listed in table 5. It will be noted that the excretion of thiamine in beriberi cases varies from 0 to 7.68 μg in children, from 0 to 8.34 μg in expectant mothers, from 0 to 8.51 μg in nursing mothers, and from 0.62 to 4.06 μg in other adults. The means for cases from all population groups were 2.85 μg for frank cases, 2.82 μg for suggestive beriberi, and 2.75 μg for doubtful beriberi.

In a study of the "fasting hour" urinary thiamine excretion of 65 apparently "normal" subjects, the excretion during the 13th hour ranged from 0.73 to 20.04 μg , with a grand mean of $5.7158 \pm 3.725 \mu\text{g}$.

The dietary intakes of these cases for a 9-day period were recorded by a procedure of keeping dietary records similar to that described in Bulletin 109 of the National Research Council ('43), and that of Phipard ('44). Under this procedure dietary histories are assessed in conjunction with the nutritional status of selected subjects. Although according to Kruse ('44) it was observed in his surveys that biochemical methods demonstrated, not the nutritional status,

TABLE 5

"Fasting hour" urinary thiamine excretion in micrograms of beriberi cases in Bataan by age, physiological condition and degree of beriberi¹

POPULATION GROUP	FRANK BERIBERI		SUGGESTIVE BERIBERI		DOUBTFUL BERIBERI		TOTAL CASES	
	Number of cases	Mean thiamine excretion	Number of cases	Mean thiamine excretion	Number of cases	Mean thiamine excretion	Number of cases	Mean thiamine excretion
		μg		μg		μg		μg
Children ² (2.01-15 years)	13	3.39	7	2.57	24	2.55	44	1.69
Expectant ³ mothers	43	4.27	74	3.14	122	2.53	239	2.64
Nursing ⁴ mothers	79	2.32	120	2.63	291	2.42	490	2.41
Other adults ⁵ (15.01 years and over)	105	2.64	230	2.66	439	2.88	774	2.61
Total beriberi cases examined	240	2.85	431	2.82	876	2.75	1,547	2.72

¹ Number of cases examined for thiamine not necessarily equal to number of beriberi cases.

² Minimum value in micrograms—0.00 (D.B.). Maximum value in micrograms—7.68 (F.B.). D.B. = doubtful beriberi and F.B. = frank beriberi.

³ Minimum value in micrograms—0.00 (F.B.). Maximum value in micrograms—8.34 (F.B.). F.B. = frank beriberi.

⁴ Minimum value in micrograms—0.00 (F.B.). Maximum value in micrograms—8.51 (S.B.). F.B. = frank beriberi and S.B. = suggestive beriberi.

⁵ Minimum value in micrograms—0.62 (F.B.). Maximum value in micrograms—4.06 (D.B.). F. B. = frank beriberi and D.B. = doubtful beriberi.

but mainly the nature of the immediate past dietary intake, the data gathered from the dietary histories in this survey were considered in relation to thiamine excretion. The values for calories and for thiamine in the various articles of the diet were obtained from the food tables prepared by Concepcion ('47). The formula of Williams and Spies ('38) of micrograms of thiamine intake divided by calories and the formula of Cowgill ('39) on the thiamine requirements of individuals were used in eliminating 15 of the original 65 apparently "normal" individuals. Ten of the 15 subjects so eliminated have values of the T/Cal. ratio below 0.25 which, according to Williams and Spies, is indicative of clinical beriberi, and they also have thiamine intake deficits according to Cowgill's formula. Five of the 15 subjects showed stray figures. The grand mean of "fasting hour" urinary thiamine excretions after the foregoing procedure became $6.0828 \pm 3.39 \mu\text{g}$.

It becomes apparent from the foregoing discussion that the values obtained for the "fasting hour" urinary thiamine excretion in beriberi cases are not diagnostic of the deficiency when considered apart from the symptoms and signs characteristic of the disease. However, the presence of symptoms and signs together with low excretions of thiamine (from 0 to 2 μg) in the "fasting hour" urine specimen is helpful in the diagnosis of beriberi. The present authors see no correlation between the excretion of urinary thiamine in the "fasting hour" and the severity of beriberi. The writers of this paper believe that the dietary history, the physical examination and the "fasting hour" urine examination for thiamine should be taken together in the diagnosis of beriberi in individuals and in the assessment of beriberi incidence in a community. We are inclined to agree with Darby ('47) that "inasmuch as variability is a fundamental attribute in biology, it becomes impossible to fix a single figure as the requirement for a substance," and we may add that it is likewise impossible to say that a given figure for a biochemical test, as such tests are developed at present, is diagnostic of beriberi.

The incidence of clinical beriberi with respect to the thiamine-calorie ratio was studied more in detail by the computation of thiamine and calories in a 9-day dietary history of beriberi subjects selected at random in Bataan Province. The results of this study are shown in table 6.

There seems to be concentration of cases in the groups below Williams and Spies' ('38) T/Cal. borderline ratio of 0.25 in which 68% of all cases, 76% of frank cases and 80% of suggestive cases occur.

TABLE 6

Incidence of clinical beriberi with respect to thiamine-calorie (T/Cal.) ratio

T/CAL.	CASES NO.	FRANK		SUGGESTIVE		DOUBTFUL		MEAN FASTING HOUR EXCRETION μ g
		no.	%	no.	%	no.	%	
0.10-0.149	7	1	14.28	3	42.85	3	42.85	2.71
0.15-0.199	34	7	20.58	7	20.58	20	58.82	3.65
0.20-0.249	43	5	11.62	10	23.24	28	65.11	2.41
0.25-0.299	21	2	9.52	2	9.52	17	80.95	3.17
0.30-0.349	10	1	10.00	3	30.00	6	60.00	1.87
0.35-0.399	6	0		0		6	100.00	2.50
0.40 up ¹	1	1	100.00	0		0		0.61

¹ Last item apparently a stray figure.

Rearrangement of the series according to an ascending order of deviation of actual thiamine intake from Cowgill's theoretical thiamine intake does not reflect any improvement on the above results as it involves only an internal shuffling of individual groups. In fact, 64.7% of all cases had no thiamine deficit, using Gowgill's formula.

Dietary practices of rice-eaters in Bataan

To complete the information given in this paper, inquiries were made about the dietary practices of rice-eaters in Bataan. It is noteworthy that 30% of the subjects studied, washed rice twice before cooking, 55% three times, and 15%

4 times. All rice washings were discarded by 81% of the subjects.

An impression of the dominance of rice in the prevailing dietaries may be gained from the following statements: Two-thirds of the families surveyed reported the use of meat only once a week; fish was consumed by 90% of the families 6 or 7 times a week; vegetables of some sort were used, by more than half the population, not more than twice a week.

SUMMARY

A clinical beriberi survey was undertaken in the Province of Bataan, Philippines, a sub-tropical area with endemic beriberi. The clinical survey included "fasting hour" urinary thiamine analysis. The population surveyed consisted of 11 to 64% of the total population in each of 9 of the 12 municipalities of the Province. A total of 12,384 persons were examined and 1,580 cases of frank or suspected beriberi were found, corresponding to 12.7% of the population. Frank beriberi occurred in 1.99%.

Beriberi is found in descending order of frequency in "expectant mothers," "nursing mothers," "other adults," "infants," and "children." However, the reported mortality from beriberi is highest in infants and then in "other adults," there being no deaths of expectant mothers and only a very small number among nursing mothers.

Discussion is presented of the frequency of signs and symptoms of clinical beriberi, of the limited usefulness of the so-called "fasting hour" urinary thiamine excretion test, and of the dietary practices of rice-eaters in Bataan.

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LIVER DAMAGE AND GROWTH IN THE RABBIT

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THREE FIGURES

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The rabbit is a potentially valuable experimental subject for nutritional research, particularly for the evaluation of forages and for the study of some minor-element deficiencies that plague the farm animal in many parts of the world. The use of the rabbit in such research has been hindered by our limited knowledge of its nutritive requirements. Our experience has been that rabbits suffer liver injury and subnormal growth both when fed a whole-milk-powder diet (fortified with iron, copper, manganese, and iodine) and when fed various purified diets. This report is a preliminary investigation into the cause and prevention of liver damage and subnormal growth in rabbits subsisting on seemingly adequate diets.

Rich and Hamilton ('40) observed liver cirrhosis in rabbits fed low-protein diets and reported that daily supplements of yeast prevented this damage. The protective factor in yeast was not identified, but the diets of the animals studied would suggest a deficiency of the labile methyl group. Blumberg, Mackenzie, and Seligson ('42) reported that the liver damage suffered by rabbits on a purified diet containing 10% casein was prevented by daily supplements of 300 mg of choline. Spellburg and Keeton ('40) reported fatty livers in rabbits and guinea pigs fed a scorbutigenic diet; they concluded that choline, lipocaic, or yeast is ineffective in preventing them. György ('44) suggested, in his review of experimental he-

patic injury, that a deficiency of the labile methyl group is not a complete answer to dietary-produced hepatic damage. Smith and Ellis ('47) observed fatty and cirrhotic livers in rabbits fed a whole-milk diet. Unpublished data (Smith, '44, '46) indicated that supplementation of the milk with members of the B complex, choline, yeast, or mineral mixtures containing Mg, Co, I, B, Cr, Pb, Zn, As, Mo, V, Se, Br, Be, Ni, Ti and F is not effective in preventing the liver damage.

Smith, Medlicott, and Ellis ('44) observed that the growth of rabbits fed a whole-milk diet supplemented with Fe, Cu, and Mn was only 60% of the growth of comparable rabbits fed a stock diet. Hogan and Hamilton ('42) reported that rabbits and guinea pigs grew normally on purified diets if supplemented with yeast or a water extract of dried liver, but that growth was subnormal on such diets supplemented with a vitamin mixture. Vivanco and Jimenez ('43) noted that the addition of fresh alfalfa enhanced growth and survival of rabbits fed a pea-flour and wheat-bran diet. Fresh grass has been reported essential in the feeding of breeding rabbits (Norfeldt, '42).

EXPERIMENTAL

The experimental animals used in the present study were Dutch rabbits bred in our stock colony. When the young were two weeks of age the stock diet in the cage was replaced with whole-milk powder fortified with a mineral mixture containing iron, copper, manganese, and iodine. The nursing dams were moved to another cage for several hours each day, where they were fed the stock diet. The restriction of the nursing litters to a supplement of whole-milk powder after they reached 14 days of age was instituted when it was observed (Smith, '44, '46) that the incidence of hepatic damage was reduced if the experimental animals had access to the stock ration in the pre-experimental period. The young were weaned at approximately 21 days of age, housed in individual wire-screened cages in a controlled-temperature room at 75°F., and fed the experimental diets ad libitum. All animals

were weighed weekly. At the completion of 70 days on the experimental regime, the surviving rabbits were sacrificed and the condition of the livers observed grossly. With the exception of the livers from rabbits on Diets 3, 4, 6, 7, and 11, all livers were sampled and at least one-half of these samples were examined microscopically. Formol fixed frozen sections, stained with Sudan IV and Sudan Black, were examined for fat infiltration. Sections fixed in Bouin's or Susa's fixatives and stained with hematoxylin and eosin (Mallory's or Masson's staining technique) were examined for extent of cellular damage and infiltration of connective tissue.

Four experiments were conducted at different times involving 23 different diets and 177 rabbits. Initially, 9 animals were assigned to a diet and, in subsequent trials, 6 to a diet. The composition of the experimental diets is shown in table 1. All supplements were incorporated into the diets and the diets stored at low temperatures ($-15^{\circ}\text{C}.$). The aqueous and alcoholic extracts were made at room temperature with a proportion of one to 8 by weight of dehydrated alfalfa meal and water. The mixture was agitated for three hours, then allowed to stand overnight, and filtered in a Buchner funnel. The filtrate was concentrated under a vacuum with the flask containing the extract in a water bath heated to temperatures between 40° to $60^{\circ}\text{C}.$ The residues were dried in an oven at $70^{\circ}\text{C}.$ The animals receiving Diet 1 (whole-milk powder fortified with iron, copper, manganese, and iodine) served in each of the 4 experiments as the control group for both hepatic damage and growth. To facilitate discussion, Diet 1 is designated as the basal diet. The remaining diets may be divided into those primarily designed to study the liver damage (Diets 2 to 12 inclusive) and those primarily designed to study growth (Diets 13 to 23 inclusive).

RESULTS

Survival on all diets was good, with an overall mortality of less than 6%. More than half of the deaths occurred on the diets compounded of purified ingredients. In general,

TABLE 1
Percentage composition of diets¹

DIET NO.	COMPOSITION
1	Milk ² 100.
2	Milk 100, vitamin supplement. ³
3	Milk 50, Ruffex ⁴ 15, starch ⁵ 33, minerals ⁶ 2.
4	Milk 50, Ruffex 15, dextrose 33, minerals 2.
5	Milk 90, alfalfa ⁷ 9.8, DL-methionine 0.2.
6	Ruffex 15, starch 40, crude casein ⁸ 20, fat ⁹ 11, yeast ¹⁰ 10, minerals 4.
7	Ruffex 15, dextrose 20, starch 20, crude casein 20, fat 11, yeast 10, minerals 4.
8	Ruffex 15, starch 40, sucrose 10, crude casein 20, fat 11, minerals 4, vitamin supplement. ¹¹
9	Ruffex 10, dextrin ¹² 36, vitamin-free casein ¹³ 25, fat 25, minerals 4, vitamin supplement. ³
10	Same as diet 9 plus cholesterol 0.15.
11	Milk 50, alfalfa 50.
12	Milk 75, alfalfa 25.
13	Milk 90, alfalfa 10.
14	Milk 95, alfalfa 5.
15	Milk 97, alfalfa 3.
16	Milk 99, alfalfa 1.
17	Milk 99.5, alfalfa 0.5.
18	Milk 97, ¹⁴ aqueous extract equivalent to alfalfa 10.
19	Milk 93, ¹⁴ residue aqueous extract equivalent to alfalfa 10.
20	Milk 99.7, ¹⁴ aqueous extract equivalent to alfalfa 1.
21	Milk 99.3, ¹⁴ residue aqueous extract equivalent to alfalfa 1.
22	Milk 99.8, ¹⁴ filtrate alcoholic precipitate of aqueous extract equivalent to alfalfa 5.
23	Milk 98.7, ¹⁴ alcoholic precipitate of aqueous extract equivalent to alfalfa 5.

¹ All diets were supplemented to contain a minimum of 10 mg Fe, 0.8 mg Cu, and 0.6 mg Mn.

² Klim, Borden Company.

³ Milligrams/100 gm: choline 200, niacin 20, inositol 10, pyridoxine 0.7, thiamine 0.7, riboflavin 0.7, calcium pantothenate 1.5, biotin 0.25, folic acid 0.25, 2 methyl, 3 phytol, 1,4-4 naphthoquinone 0.075, alpha tocopherol acetate 7.5, crystalline A 0.20, viosterol 40.

⁴ Fisher Scientific Company.

⁵ Cooked starch.

⁶ Osborne and Mendel.

⁷ Seventeen per cent dehydrated alfalfa meal, W. J. Small Company, Kansas City, Missouri.

⁸ Argentine crude casein.

⁹ Primex, hydrogenated vegetable oil.

¹⁰ Anheuser-Busch.

¹¹ Milligrams/100 gm: choline 200, niacin 20, inositol 10, pyridoxine 0.7, thiamine 0.7, riboflavin 0.7, calcium pantothenate 1.5, biotin 0.25, alpha tocopherol acetate 7.5.

¹² Merck N.F.V. white dextrin.

¹³ Labco, Borden Company.

¹⁴ Approximations.

the rabbits appeared healthy and thrifty, except those receiving Diets 9 and 10. On these two diets the rabbits were noticeably smaller, with rough, shaggy, dull coats and unthrifty appearances.

Liver injury

The essential observations on the condition of the livers of all rabbits surviving the 70-day experimental period are noted in table 2. The livers of the 24 surviving animals fed Diet 1 showed fatty and fibrous degeneration, characterized in the majority of cases by a reddish-yellow color and tenderness or slight resistance to probing: in some instances the liver was a light yellow in color, possessed a pebbled or "hob-nail" surface, and was turgid and hard. Histologically, the picture was one of early cirrhosis manifested by degeneration and destruction of hepatic cells, a regeneration of liver cells from those escaping destruction, a slight connective tissue proliferation at the portal canal, and an accompanying fatty infiltration of the liver cells (see figs. 1, 2, and 3, Plate 1). No ceroid pigment was evident. The histological studies will be presented in detail elsewhere.

The addition to the basal diet of 15% cellulose from rice bran,¹ 2% salt mixture, and 33% starch or dextrose (Diets 3 and 4) had no effect on the incidence of hepatic damage. The same result was noted when the basal diet was fortified with a "complete" vitamin mixture (Diet 2).

The livers of the animals receiving the diets compounded with purified ingredients using yeast as a source of the accessory food factors indicated slight fatty and fibrous degeneration in 9 of the rabbits, with the livers of 4 of the animals apparently normal (Diets 6 and 7). The feeding of a similar purified diet (Diet 8) with a vitamin mixture replacing the yeast resulted in all animals suffering liver damage.

Liver damage was also observed in rabbits receiving a diet formulated to approximate the composition of milk powder

¹"Ruffex," Fisher Scientific Co., Pittsburgh, Pennsylvania.

TABLE 2

Summary of results pertaining to liver condition and average body weights and gains of rabbits

DIET NO.	DESCRIPTION	NO. OF ANIMALS		LIVER CONDITION	BODY WEIGHT		Gain ¹
		Initial	Final		Final	Initial	
1 ²	Milk, 100%				gm	gm	gm
a		9	8	Fatty and fibrotic	800	189	611 ± 71
b		6	6	Fatty and fibrotic	914	246	668 ± 112
c		5	5	Fatty and fibrotic	820	268	552 ± 117
d		6	5	Fatty and fibrotic	909	210	699 ± 123
2	Milk 100% + vitamins	6	6	Fatty and fibrotic	956	211	745 ± 67
3	Milk 50%, Ruffex, starch	9	9	Fatty and fibrotic	748	238	510 ± 46
4	Milk 50%, Ruffex, dextrose	9	9	Fatty and fibrotic	637	208	429 ± 48
5	Milk 90%, alfalfa 9.8%, methionine 0.2%						
		6	6	Fatty and fibrotic	1073	293	780 ± 89
6	Purified + yeast 10%	9	7	5 fatty, 2 normal	893	230	663 ± 32
7	Purified + yeast 10%	9	6	4 fatty, 2 normal	867	202	665 ± 82
8	Purified + vitamin supple- ment	6	4	Fatty and fibrotic	815	266	549 ± 29
9	Purified + vitamin supple- ment	6	6	Fatty and fibrotic	561	172	389 ± 44
10	Diet 9 + cholesterol 0.15%	6	6	Fatty and fibrotic	487	190	298 ± 64
11	Milk 50%, alfalfa 50%	9	9	Normal	1181	242	939 ± 72
12	Milk 75%, alfalfa 25%	6	6	Fatty, fibrotic	1173	205	968 ± 91
13	Milk 90%, alfalfa 10%	6	6	Fatty, fibrotic	1350	252	1098 ± 66
14	Milk 95%, alfalfa 5%	6	6	Fatty, fibrotic	1176	217	959 ± 82
15	Milk 97%, alfalfa 3%	6	6	Fatty, fibrotic	1099	181	918 ± 82
16	Milk 99%, alfalfa 1%	6	5	Fatty, fibrotic	1259	225	1034 ± 61
17	Milk 99.5%, alfalfa 0.5%	6	6	Fatty, fibrotic	795	204	591 ± 85
18	Milk 97%, water extract equiv. to 10% alfalfa	6	6	Fatty, fibrotic	1037	288	749 ± 104
19	Milk 93%, residue water ex- tract equiv. to 10% alfalfa	6	6	Fatty, fibrotic	1146	277	869 ± 153
20	Milk 99.7%, water extract equiv. to 1% alfalfa	6	6	Fatty, fibrotic	975	252	723 ± 95
21	Milk 99.3%, residue water extract equiv. to 1% alfalfa	6	6	Fatty, fibrotic	1114	255	859 ± 40
22	Milk 99.8%, alcoholic fil- trate water extract equiv. to 5% alfalfa	6	6	Fatty, fibrotic	772	178	594 ± 76
23	Milk 98.7%, alcoholic pre- cipitate water extract equiv. to 5% alfalfa	6	6	Fatty, fibrotic	1007	234	973 ± 58

¹ With standard errors.² Statistical comparisons with respect to gains in body weight: **Highly significant ($P < 0.01$); *significant ($P < 0.05$). (For detailed discussion see text.):

Diet 1a with diets 3, 4, 6, 7, and 11**

Diet 1b with diets 8 and 13**

Diet 1c with diets 18 and 19

Diet 1d with diets 2, 9, 10, 12, 14, 15, 16*, 17, 20, 21, 22, and 23

Diet 8* with diet 9

in its content of protein, carbohydrate, and fat, and supplemented with a vitamin mixture (Diet 9). When 0.15% cholesterol was added to this diet (Diet 10), the severity of the hepatic damage was not affected.

When Diet 1 was supplemented with dehydrated alfalfa meal at the 50% level, liver damage was not observed (Diet 11). No protection from liver damage was afforded the animal if this level of supplementation with alfalfa meal was reduced to 10% (Diet 13) or to 25% (Diet 12).

Methionine (0.2%) exhibited no lipotropic action according to histological evidence when added to the basal diet containing approximately 10% dehydrated alfalfa (Diet 5).

Growth

In table 2, the average initial and final live weights and the average gain in body weight with its standard error are given for the surviving rabbits on all diets studied. Since the various diets were studied in 4 different experiments with a control group of animals receiving Diet 1 in each experiment, the average data for the control groups are given separately as Diet 1a, b, c, and d. Statistical comparisons were made only within experiments. As may be noted from the standard errors, the individual variation within groups, particularly those fed Diet 1, is large. Random breeding resulting in variable weaning weights and growth potentials was a contributing factor to the variation within the groups. The greater variability on the basal diet may be accounted for by the fact that severe liver damage occurred in one or two individuals in each of the groups on Diet 1. Apparently incipient liver damage does not affect growth under our experimental conditions, but the progression of this damage to a cirrhotic condition does influence growth. In the 24 surviving rabbits which received Diet 1, 6 were observed to have suffered severe liver damages and all 6 showed either a decreased growth rate or a cessation of growth and a loss of body weight.

Although the obvious corrective action for large variability is to increase the number of animals, it was felt that in preliminary studies of this nature with a limited number of animals statistical precision could be sacrificed in favor of a more comprehensive survey and study of the nutritional abnormalities encountered in the rabbit subsisting on a diet of mineralized whole milk powder. Statistical significance was determined by the "t" value as calculated from the body weight differences and their standard errors. Odds greater than 19:1 are accepted as being significant, and odds greater than 99:1 are considered highly significant.

The addition of 50 and 10% supplements of alfalfa meal to the basal diet (Diets 11 and 13) resulted in a highly significant increase in body weight when compared to the gain in body weight of the respective control animals (Diets 1a and 1b), but only the 50% supplement prevented liver damage. When dehydrated alfalfa was fed at a level of 1% (Diet 16), a significant increase in body weight was observed when compared to the gains made by the rabbits receiving Diet 1d. The supplementation of the basal diet with alfalfa at levels of 25, 5, 3, and 0.5% (Diets 12, 14, 15, and 17) did not result in significantly increased gains as compared with gains made by the control animals (Diet 1d). If the 24 rabbits receiving the basal diet are treated statistically as a group (the average gain and its standard error would be 631 ± 49), all supplements of alfalfa meal with the exception of Diet 17 (0.5% alfalfa) resulted in a highly significant increased gain in body weight.

The feeding of water extracts and their residues as supplements to the basal diet at levels equivalent to 10 and 1% alfalfa (Diets 18, 19, 20, and 21) did not significantly stimulate growth when compared with the growth obtained in the respective control groups (Diets 1c and d). The supplementation of the basal diet with the filtrate and residue of an 80% alcoholic precipitation of an aqueous extract of alfalfa at a level equivalent to 5% alfalfa (Diets 22 and 23) did not

result in a body gain significantly greater than that of the control group (Diet 1d).

The significantly greater gain in weight of the animals fed Diet 8 compared to the body gain of the rabbits fed Diet 9 is of interest. Both diets were formulated from purified ingredients and supplemented with vitamin mixtures.

DISCUSSION

The results presented here (Diets 2, 5) indicate that the hepatic damage suffered by rabbits fed solely on a mineralized milk diet is not caused by a deficiency in this diet of the known members of the vitamin B complex, of the fat-soluble vitamins, or of the labile methyl group. That the liver damage is not caused by lack of roughage or by toxic constituents in the milk is indicated by a comparison of Diets 3, 4, and 11. In each of these diets milk made up to 50% of the mixture, yet the animals fed on Diets 3 and 4 suffered liver injury whereas the livers of the rabbits receiving Diet 11 were normal. Several authors have reported that liver damage may be produced in the rabbit by feeding cholesterol (Chalatow, '14; Baily, '16). However, the inclusion of cholesterol in a purified diet at a level comparable to the cholesterol concentration in whole-milk powder failed in the present case to increase the severity of the liver damage.

According to our results, then, dehydrated alfalfa seemingly contains an unidentified liver-protecting substance, but this protective substance seems to be present in relatively low concentrations since a 10 or 25% "alfalfa-milk" diet does not prevent liver damage.

We have also observed that the inclusion of 10% yeast² in purified diets partially protected the rabbit from liver damage. Rich and Hamilton ('40) reported that 5 gm of brewers' yeast fed daily as a supplement to a low-protein diet prevented the development of cirrhotic livers in rabbits. Spellburg and Keeton ('40) found that guinea pigs and rab-

² Anheuser-Busch.

bits suffered fatty degeneration of the liver on 30% whole-milk diets, but that yeast³ was not protective at the 1% level.

The observation (Smith, '44-'46) that if the experimental animal was permitted to consume stock ration before weaning, liver damage was not invariably produced on the milk diet suggests that either (1) the animal was able to store sufficient quantities of the factor before being placed on the milk diet, or (2) a bacterial flora favorable for the production of the factor was well established in the brief pre-weaning consumption of stock ration. In our studies, the addition of dextrose or starch to the milk diet had no effect on the observed liver damage, nor did the formulation of purified diets with various carbohydrates.

Also of interest in connection with the problem of liver damage, produced by dietary means, which does not respond to the recognized lipotropic factors are the reports of Schwarz ('44a, '44b). This author observed liver damage in rats fed purified diets containing alkaline-washed casein which could be prevented by supplements of wheat germ oil, cream, sunflower seed oil, total lipids of adrenal gland, xanthine, or a 20-fold increase of alpha tocopherol over the normal requirement. Other workers (Forbes, '39) have reported that xanthine and xanthine-containing preparations prevent liver damage in rats subjected to carbon tetra-chloride poisoning.

The increase in body weight of young rabbits fed a basal milk diet is markedly stimulated with supplements of dehydrated alfalfa meal at a level as low as 1%. Kohler, Elvehjem and Hart ('36, '37) report increased growth in rats fed a "winter milk" diet supplemented with fresh grass, dried grass, or grass juice. These workers have also shown that dehydrated grass or grass juice is essential for the nutrition of the guinea pig fed a "winter milk" ration (Kohler, Elvehjem, and Hart, '38; Kohler, Randle, Elvehjem, and Hart, '39). It seems possible that the growth factor in dehydrated grass and grass juice essential in the nutrition of the rat and guinea pig is also necessary in the nutrition of the rabbit. Our fail-

³ Fleischman's.

ure to extract with water the growth factor from dehydrated alfalfa in appreciable quantities is in agreement with the findings of Kohler, Randle, and Wagner ('39), who reported that the "grass juice factor" could not be extracted from dehydrated grass with water.

The differences in the growth-promoting properties of the purified diets varying in their contents of protein, carbohydrates, fat, roughage, vitamin supplements, and source of casein suggest the existence of another nutritional factor required by the rabbit. Since animals receiving the diet furnishing more protein and energy per gm of food and larger vitamin supplements gained the least in body weight, it would seem unlikely that the differences in composition and in vitamin supplements account for the differences in body gains. The protein of the diet associated with the larger gain in body weight was crude casein, while the protein of the other diet was a purified vitamin-free casein. This suggests that crude casein contains a factor essential for the nutrition of the rabbit. This postulated rabbit-growth factor in crude casein may be similar to strepogenin, which is reported to be a growth factor for the guinea pig (Wooley and Sprince, '45), and for which crude casein is a more potent source than purified casein; or, more likely, it may be similar to the animal protein factor said to be essential for normal growth in the rat (Cary et al., '46) and in the chick (Coombs, '47).

SUMMARY

A diet of whole-milk powder (fortified with manganese, copper, iron, and iodine) is inadequate for the rabbit in at least two respects. Growth is subnormal and the livers are fatty and cirrhotic. Large amounts (50%) of dehydrated alfalfa meal are needed to prevent liver injury, while only small amounts (1%) are needed to stimulate the growth rate. Experiments involving 23 diets and 177 animals indicate that milk does not contain a toxic factor responsible for these results, and that the results are not due to the lack of rough-

age or of any of the known fat- and water-soluble vitamins or of the labile methyl groups. Preliminary attempts to extract the growth-promoting factor from alfalfa with water were unsuccessful.

Purified diets when supplemented with a complete vitamin mixture offered no protection against liver damage, but when supplemented with yeast offered partial protection. In these diets crude casein produced better growth than purified casein, indicating the need of the rabbit for a third factor, possibly similar to the "animal-protein factor" of Cary et al. ('46) and Coombs ('47).

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PLATE 1

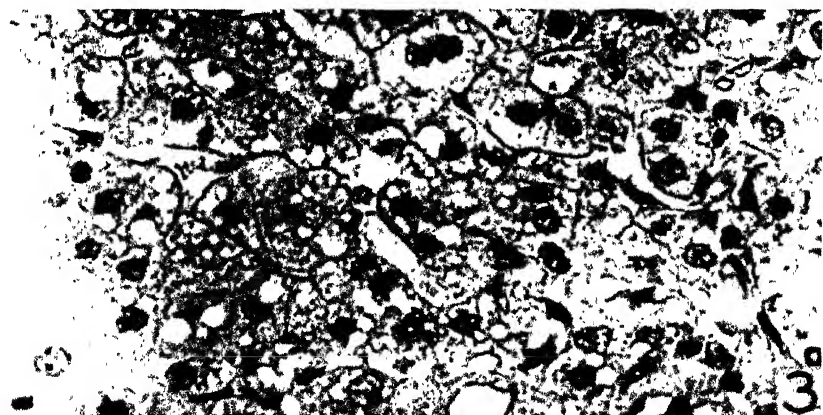
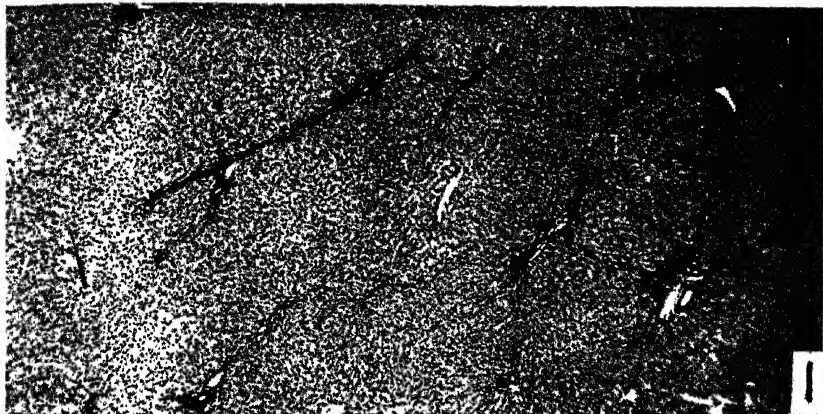
EXPLANATION OF FIGURES

Photomicrographs of damaged liver sections fixed in Susa's fixative, sectioned, and stained with hematoxylin and eosin.

1 This picture shows the slight proliferation of connective tissue originating at the portal canal as usually seen in the damaged livers (approx. 60 \times).

2 The light areas indicate hepatic cell destruction accompanied by the infiltration of connective tissue (approx. 120 \times).

3 The vacuolated appearance of these hepatic cells indicates the areas from which fat was removed in the preparation of the tissue for sectioning and staining (approx. 600 \times).



THE RELATIVE BIOLOGICAL VALUES OF THE PROTEINS IN CEREAL GRAINS¹

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Mitchell ('24a) found that on the same level of protein intake (5%) the proteins in whole brown rice are superior to those in corn and oats. Recently, while engaged in studies of the influence on growth and protein utilization of additions of small amounts of cultured food yeasts to the proteins in cereal flours and cereal grains, it was observed that, on the same protein level in the rations (5.8%) the proteins in polished rice are far superior to those in enriched milled wheat flour (Sure, '46, '47), as evidenced from gains in weight per gm of protein intake.

The purpose of this investigation was to determine the relative biological values of the proteins in rice, wheat, rye and corn, in both the milled and unmilled states². Because of the low nitrogen content of rice, it was necessary to feed these cereal grains in such proportions as to introduce into the rations a total of not more than 5% of proteins.

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² The whole wheat, whole rye, and milled rye were furnished by General Mills, Inc., Minneapolis, Minn. The whole corn and milled corn (white table meal) were furnished by the Staley Manufacturing Co., Decatur, Ill. The whole brown and milled or polished rice was obtained from the Walton Rice Mills, Stuttgart, Ark. The milled wheat was Gold Medal (General Mills, Inc.) enriched flour, and the rolled oats were a Quaker Oats oatmeal product.

The biological values of the proteins of the cereal grains were determined by the nitrogen balance method (Mitchell, 24b, '44), in which the values are generally expressed as the per cent of absorbed nitrogen retained by the animal. Wistar strain albino rats were used and in all groups the sexes were equally divided. The ration fed was a modification of the one suggested by Mitchell and Hamilton,³ which had the following percentage composition: Cellu flour, 2; lard, 10; Sure's salts no. 1 (Sure, '41), 4; cod liver oil, 1.5; wheat germ oil, 0.5; enough cereal grains to furnish 5% protein; and the balance, cerelese. The following components of the vitamin B complex were administered daily to each animal separately from the ration: 50 μ g each of thiamine, riboflavin, pyridoxine, and nicotinic acid; 300 μ g calcium pantothenate; 9 mg choline chloride; 6 mg *p*-aminobenzoic acid, and 2 mg inositol.

The nitrogen metabolism experiments were generally conducted on 24 animals, 12 males and 12 females, so that using 8 animals for each group, it was possible to study three cereal grains at one time. However, occasionally the results on one or two rats deviated so much from the rest of the group that it was necessary to eliminate such data from the averages; hence, data in some cases are shown for 6 animals. In the case of the rice experiments, it was necessary to use more animals in order to obtain reliable average figures.

Urinary and fecal balances were carried out for 7 days on an egg standardizing ration and 7 days on the experimental rations containing the various cereal grains. In each case the animals were allowed to get accustomed to consuming the entire rations for a pre-test period of from three to 5 days before the beginning of the nitrogen balance studies. The percentage composition of the egg ration is as follows: Dried defatted whole egg, 5.8; cellu flour, 2.0; Sure's salts no. 1, 4; cod liver oil, 1.5; wheat germ oil, 0.5; lard, 10.0; and cerelese, 76.2. The dried whole eggs were defatted by repeated extractions with large volumes of petroleum

³ Personal communication.

ether at room temperature. The defatted dried whole eggs contained 69.2% protein and furnished 4.0% protein in the ration. Feces markers were used. The fiber contents of the diets were not equalized.^{*} However, the slight errors produced in measurements of fecal nitrogen excretions by neglect to balance the fiber contents of the standardizing diet and the test diets would be approximately the same for all the milled cereal grains and all the whole cereal grains, and would not influence findings as to the comparative biological values of the milled and unmilled cereal grains fed at the 5% protein level.

Our findings are summarized in table 1. It is evident from this table that when the milled cereal grains are fed as the only sources of proteins in the ration, at a 5% level of protein intake, the milled corn (as sold in grocery stores under the name of "table meal") is by far the poorest source and the milled rice is the best. There are no marked differences in the protein values of the milled wheat and milled rye. The net protein utilization values of the whole cereal grains are very similar. It will be noted that although the animals which received the whole corn as the only source of protein in the ration consumed about 40% less food than those which were fed the milled table meal, they utilized their proteins over twice as well. It would appear, then, that from the protein standpoint, the corn receives too drastic treatment in the course of being milled. Apparently too many bran layers, which carry lysine and tryptophane, the amino acids most deficient in the corn kernel (Mitchell and Smuts, '32), are removed. Recently it was demonstrated that the proteins in table corn meal are materially improved by the addition of such amino acids (Sure, '48).

As a control experiment, the biological value of lactalbumin,⁴ was determined at a 5% protein level. The true digestibility was found to be 100%, and the biological value, 93.0%; therefore, the net utilization was also 93.0.

^{*} Borden.

TABLE 1
The relative biological values of the proteins in cereal grains fed at a 5% level of protein intake

CEREAL GRAIN OR FLOUR	BODY WEIGHT	FOOD INTAKE	N ¹ INTAKE	FECAL N	META- BOLIC N IN FECES	FOOD N IN FECES	AB- SORBED N	N IN URINE	META- BOLIC N IN URINE	FOOD N IN URINE	FOOD N RE- TAINED	TRUE DIGESTI- BILITY	BIO- LOGI- CAL VALUE ²	NET UTILI- ZATION ³
	gm	gm	mg	mg	mg	mg	mg	mg	mg	mg	%			
Milled rice (14) ⁴	59.9	7.4	65.2	11.4	8.3	3.1	60.1	26.6	14.1	12.5	47.6	95.1	79.0	75.1
Milled wheat (8)	53.3	6.9	59.5	10.4	7.4	3.0	56.5	32.4	11.7	20.7	35.8	94.5	63.5	60.0
Milled rye (8)	62.6	7.6	65.3	14.0	8.1	5.9	59.4	33.1	15.1	18.0	41.4	90.5	69.7	63.1
Milled corn (8)	83.0	6.2	38.1	11.6	7.2	4.4	33.7	42.1	16.2	25.9	12.2	88.5	36.2	32.0
Whole rice (14)	59.4	4.8	44.2	7.6	5.0	2.6	41.6	21.3	15.1	6.2	35.4	94.1	85.1	80.0
Whole wheat (8)	60.2	5.0	43.6	9.5	5.9	3.6	40.0	23.9	17.1	6.8	33.2	91.7	83.0	76.1
Whole rye (6)	55.3	7.8	66.8	14.8	6.7	6.1	60.7	29.0	17.1	11.9	48.8	91.0	80.4	73.2
Whole corn (8)	53.7	3.9	30.9	6.5	4.4	2.1	28.8	19.7	15.1	4.6	24.4	93.0	84.7	78.8
Rolls oats (6)	60.5	7.6	65.4	11.8	6.6	5.2	60.2	27.3	16.5	10.8	49.4	92.1	82.1	75.6

¹ N = Nitrogen.

² Per cent of absorbed nitrogen retained in the body.

³ The value for the true coefficient of digestibility multiplied by the biological value, divided by 100.

⁴ Numbers in parenthesis indicate the number of animals used in the nitrogen balance experiment.

The results submitted in this paper are in harmony with the observations of Osborne and Mendel ('20), who reported that there were no notable differences in growth of albino rats when the entire oat, rye, and wheat kernels were used as the only sources of protein in their rations; and are also in agreement with the recent findings of Jones, Caldwell, and Widness ('48), who found from growth experiments that at the low 4.5% protein level, corn, hard wheat, soft wheat, barley and rice in the milled state showed essentially the same values. The higher biological values for whole or brown rice than for milled or white rice are in agreement with the results of Kik ('32) but not in accord with those of Jones and associates ('48). As the protein level is decreased, the efficiency of protein utilization is increased; hence our results on biological values for the proteins in all the whole cereal grains are higher than are reported in the literature concerning corn, wheat, and rye, which have been studied for 8 and 10% levels.

Since corn is used widely as a source not only of calories but also of protein and vitamins by people of low income levels in the South, it would seem that there is a need for undermilling of corn, at least to the extent practised with wheat, rice, and rye, so as to leave in the milled product proteins of considerably higher biological value.

SUMMARY

The biological values of milled and whole cereal grains, fed at a 5% protein level, were determined by the Mitchell ('24b, '44) nitrogen balance method. The protein utilization for the milled cereal grains was as follows: Rice, 75.1; wheat, 60.0; rye, 63.1; and corn, 32.0. The findings for the whole cereal grains were: Rice, 80.0; wheat, 76.1; rye, 73.2; corn, 78.8; and rolled oats (oatmeal), 75.6.

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PTEROYLGLUTAMIC ACID REQUIREMENT OF THE RAT AND A CHARACTERISTIC LESION OBSERVED IN THE SPLEEN OF THE DEFICIENT ANIMAL^{1,2}

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TWO FIGURES

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The deficiency syndrome in young rats caused by lack of pteroylglutamic acid (PGA) was first described by Black, McKibbin and Elvehjem ('41). Later, Spicer, Daft, Sebrell and Ashburn ('42) made a detailed study of this deficiency, showing that it is characterized not only by a cessation of growth but also by leukopenia, granulocytopenia, bone marrow hypoplasia and, occasionally, anemia. These same investigators observed that the blood dyscrasia and cessation of growth can be largely prevented or successfully treated with whole dried liver extract or with certain fractions of liver extract. Today, these substances are known to contain appreciable amounts of PGA.

In order to induce PGA deficiency in the rat, it is necessary to feed succinylsulfathiazole (SST) or some other inhibitor

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²A preliminary report of this investigation was presented by the author at the meeting of the American Federation of Biological Societies held at Atlantic City, March 15-19, 1948.

together with the deficient diet, thus depriving the animal of the PGA that is known to be synthesized by its normal intestinal microflora.

The present paper is concerned with the determination of the pteroylglutamic acid level necessary to restore normal growth, white cell count, and spleen morphology to PGA-depleted rats.

EXPERIMENTAL

One hundred and fifty-three 21-day-old Wistar albino rats from our colony were kept in cages with raised wire floors, three to a cage. The basal purified diet free of PGA fed ad libitum to these animals had the following composition per kg: sucrose, 599 gm; vitamin-free casein,³ 180 gm; salt mixture,⁴ 40 gm; cellu flour, 40 gm; hydrogenated vegetable oil,⁵ 100 gm; corn oil,⁶ 20 gm (containing 52,000 I.U. vitamin A, 13,000 I.U. vitamin D, and 32 mg tocopherol); succinylsulfathiazole, 20 gm; choline chloride, 1 gm; inositol, 8 mg; nicotinic acid, 40 mg; calcium pantothenate, 44 mg; thiamine hydrochloride, 8 mg; riboflavin, 16 mg; pyridoxine hydrochloride, 8 mg; *p*-aminobenzoic acid, 4 mg; biotin, 1 mg;⁷ 2-methyl-1,4-naphthoquinone, 10 mg.

As far as we have been able to determine,⁸ our rat colony is free from *Bartonella* parasites.

³ General Biochemicals, Inc.

⁴ Sodium chloride, 4.35%; magnesium sulfate, 13.70%; sodium biphosphate, 8.72%; potassium phosphate (K_2HPO_4), 23.98%; calcium biphosphate $CaH_4(PO_4)_2 \cdot H_2O$, 13.58%; ferric citrate (U.S.P. reagent, 17.5%, Fe), 2.97%; calcium lactate, 32.70%.

⁵ Spry.

⁶ Mazola.

⁷ Provided by Dr. Elmer L. Sevringhaus of Hoffman-La Roche, Inc., Nutley, N. J.

⁸ Smears of the blood of several animals, taken at random, were made at different intervals after splenectomy. In not a single case under observation were *Bartonella* parasites found in the blood. These animals were kept for several months and all of them gained weight at a normal rate. Their hemoglobin and red cell values were always within normal limits. Our thanks to Prof. William H. Taliaferro for examining the blood smears.

RESULTS

Induction of deficiency

The 21-day-old animals gained weight at a diminishing rate for three consecutive weeks and finally, between the third and 4th weeks on the basal diet, ceased growing altogether. Up to the third week all animals survived, but by the end of the 4th week only 91.2% of the initial number remained alive. At this time the average white cell count of the surviving animals was 3,635 cells per mm³, and all the rats began suffering from alopecia with an average rating of 2.7.⁹ Brownish cake formations on the ears, nose, and paws, as well as very soft feces, were characteristic conditions observed in practically all the depleted animals.

The average initial weight (21 days old) of the whole group of rats used was 32 gm (the group was composed of 81 males and 72 females), while the average weight of the 91.2% which survived the 4-week depletion period was 59 gm.

When autopsy was performed on the 24 animals that died between the third and 4th weeks, the only gross pathology observed in all was a yellowish distended intestine; infarcts appeared in the spleen of 73.5%.

Responses to different levels of pteroylglutamic acid

Every depleted animal was placed in an individual cage, after which 6 groups were formed of approximately the same numbers of males and females. One group did not receive the PGA supplement and served as a negative control group, while the other 5 were given, 6 times per week, in a separate dish, different levels of PGA in the form of its sodium salt.

The supplementation period lasted 5 weeks; that is, from the time the animals were 7 weeks old, when they were fully depleted, to their 12th week of growth. Weekly records were kept of their weights and of the food consumed. Before the

⁹ We used the following alopecia rating: Normal—1; mild alopecia—2; severe alopecia—3; total alopecia—4.

supplement was started, white cell counts were performed in all. These counts were repeated at the end of the 5-week experimental period or as soon as an animal showed signs of impending death. Autopsies were performed in all animals. Again, the only gross pathology consistently observed in the negative controls, and in the rats receiving suboptimal levels of pteroylglutamic acid, was the presence of splenic infarcts and of a distended intestine of a yellowish color. Records

TABLE 1

*Effects of various levels of pteroylglutamic acid (PGA) on depleted rats
(5-week supplementation period)*

PGA SUPPLEMENT	NUMBER OF ANIMALS	AVERAGE TIME OF SURVIVAL	AVERAGE GAIN OR LOSS IN WEIGHT	AVERAGE BASAL DIET CONSUMED PER ANIMAL PER DAY	AVERAGE GAIN OR LOSS IN LEUKO- CYTES	AVERAGE INCIDENCE OF IN- FARCTS IN THE SPLEEN	AVERAGE DEGREE OF ALO- PECIA AT DEATH ¹
μg		days	gm	gm	cells/mm ³	%	
None (negative) (controls)	56	5.0	— 9.0	1.6	— 1755	81.8	3.0
0.25	13	25.2	+ 21.0	4.0	— 457	50.0	3.3
0.50	17	33.0	+ 28.0	4.0	+ 72	66.0	2.4
1.25	20	29.7	+ 53.0	4.5	+ 1710	5.0	1.6
5.00	16	34.5	+ 79.0	6.3	+ 3955	0	1.5
20.00	7	35.0	+ 106.0	8.5	+ 7050	0	1.0

¹ Alopecia rating: normal = 1; mild alopecia = 2; severe = 3; total = 4.

were kept of the degrees of alopecia observed in the different groups of animals. In table 1 we have summarized the results of this experiment, and figure 1 shows the corresponding growth curves.

The negative controls lived for an average of 5 days, losing only 9 gm of weight in that time. Prior to death, their average white cell count was 1,880 cells per mm³, that is, 1,755 cells less than at the start of the supplementation period. The inci-

dence of infarcts in the spleen in the negative controls was 81.8%. All of them showed a severe alopecia.

Although the group receiving 0.25 μg of PGA lost a considerable number of white cells during the supplementation period, they did show some positive growth. They also had a high incidence of infarcts in the spleen and their alopecia index was higher than that of the negative controls, probably owing to the fact that they survived for a longer period of time and could thus develop a more severe alopecia.

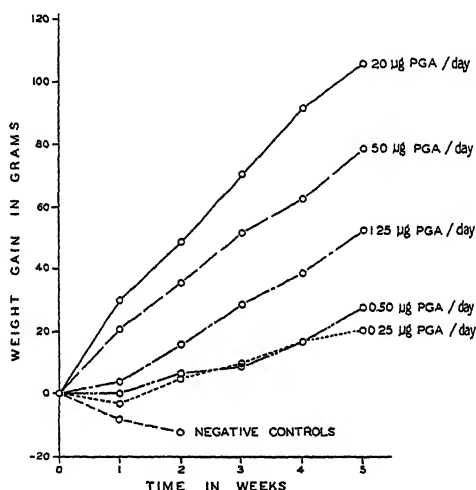


Fig. 1 Growth curves of PGA-depleted rats which received different levels of PGA 6 times per week for a period of 5 weeks.

The animals which received 0.5 μg of PGA made better gains in weight than those on 0.25 μg and gained few leukocytes during the supplementation period. The incidence of infarcts in the spleen was higher in this last group. On the other hand, the period of survival lasted 33 days while those on 0.25 μg of PGA survived for 25.2 days only.

The next group, on 1.25 μg PGA, gained 53 gm during the 5-week supplementation period — a better, but still subnormal, gain. However, they exhibited a very significant decrease in the incidence of infarcts; only one animal out of the 20

which composed the group, or 5%, showed this pathology. This level of PGA induced some tangible regeneration of the white cells, but at a subnormal rate. For this reason, at the end of the supplementation period, these animals were still suffering from leukopenia, as their average white cell count of only 5,345 cells per mm^3 showed.

The next group received 5 μg of PGA and were found, according to our standards, in a normal condition at the end of the 5-week supplementation period. Their weight gains as well as their white cell counts were within the lower limits of what we consider normal growth and normal leukocyte counts for animals of that age in our rat colony. None of the animals in this group suffered from infarcts in the spleen.

As can be seen in table 1, the rats fed 20 μg of PGA were completely normal at the end of the supplementation period.

Food intake

As the negative controls consumed only 1.6 gm of basal ration per day, the thought could not be discarded that the infarcts observed in the spleens of these animals might be the result of some secondary lack, induced by the small intake of food rather than by the PGA deficiency *per se*. This was partially ruled out by the fact that in the case of the animals on 0.25, 0.5 and 1.25 μg of PGA the incidence of infarcts in the first two groups was above 50%, while in the last group it was only 5% even though the intake of food was almost identical in the three groups; that is, 4, 4, and 4.5 gm of basal diet per day, respectively. However, to rule out completely the possibility that the low intake of basal diet was responsible for the infarcts observed, we put 6 depleted animals on 5 μg of PGA 6 times per week but restricted their basal diet intake to only 2 gm daily. These animals lost weight, the average loss being 27 gm, but all survived the maximum length of time that the negative controls had lasted, namely two weeks. The white cells rose to such a level that the animals had a normal count at the end of two weeks on the supplement.

The average gain in leukocytes was 4072 cells per mm.³ The animals were in good condition as regards cleanliness and activity. None showed lesions either in the spleen or anywhere else at autopsy 16 days after supplementary feeding was started. This was a very different picture, indeed, from that observed in the negative controls, which were consistently clumsy, slow, untidy, and suffered from a severe leukopenia and a high incidence of infarcts in the spleen. Both groups, however, exhibited severe alopecia. For the experimental group the average degree of alopecia at the end of the experiment was 3.

Splenic infarcts

None of the animals which received 5 µg, or more, of PGA per day showed infarcts in the spleen at the time they were sacrificed; that is, after receiving the supplement for 5 consecutive weeks. However, as 73.5% of the rats which died between the third and 4th weeks of depletion developed infarcts in the spleen, it is logical to conclude that a high percentage of the animals surviving the 4th week of the depletion period were already suffering from such lesions. This was partly confirmed by examining the spleens of 6 depleted rats that appeared very active and lively at the end of the 4th week. These animals were sacrificed and infarcts found in 4, while the spleens of the remaining 2 appeared normal. As it is possible that a high percentage of the depleted animals were suffering from infarction at the time supplementary feeding began, one cannot discard the possibility of regeneration taking place in the spleens of some of these animals during the supplementation period. Bloom and Taliaferro ('38) have reported complete regeneration of infarcts in the spleens of canaries caused by heavy infection with *P. cathe-merium*. In order to confirm fully this indirect observation made by us on rats, we are now conducting experiments in which we are endeavoring to ascertain, by laparotomy and by microscopical examination of the tissues, whether regen-

eration has taken place in the spleens of depleted animals treated with PGA.

The infarcts (see fig. 2) found in the spleens of animals suffering from PGA deficiency can be classified into three general groups: (1) Total infarction, where all of the organ has been affected; (2) multiple surface infarcts, in which small concentrated areas of infarction can be seen distributed



Fig. 2 Severely infarcted spleen from a rat suffering from PGA deficiency. Two and $\frac{3}{4}$ times natural size.

through the surface of the organ; (3) terminal infarcts affecting either one end or both ends of the spleen.

Multiple surface infarcts were the most common of the three, while complete infarction was not seen very often. The character and intensity of infarction observed in the individual animals did not seem to depend on the level of PGA administered, as all three types of infarcts were observed in the negative controls as well as in the animals receiving sub-optimal levels of PGA. Although PGA did not seem to in-

TABLE 2

Weights of the spleens of normal and of PGA-deficient rats

	SPLEENS WITHOUT INFARCTS			SPLEENS WITH INFARCTS		
	No. of spleens	Ave. weight of spleen	Wt. of spleen per body wt.	No. of spleens	Ave. weight of spleen	Wt. of spleen per body wt.
		<i>gm</i>	<i>gm</i>		<i>gm</i>	<i>gm</i>
Normal animals (8 weeks old)	65	0.5371	0.0048
Normal animals (3 to 4 weeks old)	5	0.1919	0.0046	.	.	.
Depleted rats receiving as supplement 5 μ g PGA per day, but with the basal diet re- stricted to 2 gm per day (9 weeks old)	6	0.1639	0.0036	
Depleted animals re- ceiving as supple- ment an average of 0.5 μ g PGA per day (9 to 18 weeks old)	10	0.1449	0.0018	6	0.1422	0.0025
Negative control animals (7 to 9 weeks old)	13	0.0820	0.0019	34	0.1342	0.0031

fluence the type of infarct developed, it had a decided influence on infarct prevention and also, possibly, on its cure, as has been already pointed out.

Histological examination¹⁰ of the spleens of rats with infarcts revealed the necrotic portions of the spleen to be sharply demarcated from the more normal but intensely congested parts. In many of the sections, the splenic veins were occupied by material that probably represented a thrombus.

Splenomegaly was rarely encountered. Table 2 summarizes the data on spleen weights of normal and deficient animals.

¹⁰ We are indebted to Dr. Enrique Koppisch, of the Department of Pathology of the School, for the histological examination of the spleens. A detailed report of the histopathology of the rat in PGA deficiency is being prepared and will be published elsewhere by Koppisch et al.

The weight of the spleen computed per unit weight of animal also appears in this table.

The total weight of the spleen, or its weight per unit weight of animal, was much higher in the case of normal animals than in the case of deficient ones. The average deficient animal suffered from atrophy of the spleen. This condition was more noticeable in the case of those negative controls which did not develop infarcts than in those whose spleens suffered from this lesion.

*Induction of spleen infarction by methyl folic acid*¹¹

The basal diet used in this part of the present experiment had a composition similar to that of the previous ration, except that for the 2% of SST was substituted 0.85% methyl folic acid and the difference made up with sucrose. This diet was provided ad libitum to 18 rats, 21 days old. These animals made small gains in weight during a period of three weeks but then lost weight abruptly and died. They became very untidy, developed lesions in the skin, exhibited a severe alopecia, suffered from an acute leukopenia and also developed the ulcerative lesions in the central parts of the anterior surface of the tongue described by Franklin et al. ('47). In addition, 89% of these animals exhibited infarction of the spleen. Altogether the deficiency symptoms were much more dramatic than those observed in the animals which received the diet containing SST as inhibitor. The tongue lesions, however, have also been observed by us in some of the depleted rats receiving SST as inhibitor.

DISCUSSION

Taking as criteria of normalcy the rate of gain in weight, the concentration of white cells in the blood, and the condition of the spleen, it can be stated that the minimum amount of PGA required by depleted rats to regain their normal state

¹¹ Provided by Dr. T. H. Jukes of Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York.

is somewhere in the neighborhood of 5 μ g per day, when fed 6 times weekly during a 5-week period. Those animals which received 20 μ g per day gained weight and recovered white cells at an optimum rate, and not a single one suffered from spleen infarction.

The level of 5 μ g of PGA per day, found by us to be the minimum requirement of depleted rats for normalcy, is within the range obtained recently by another investigator (Welch, '48) in the course of some exploratory experiments. He finds that the minimum amount of PGA required per day by depleted rats is somewhere between 2 and 10 μ g.

As far as we can learn from the literature, the above observations regarding the development of infarcts associated with pteroylglutamic acid deficiency in rats have not been previously described. That the spleen infarction observed is a direct result of PGA deficiency seems to be well substantiated by the fact that at increased levels of PGA incidence decreased, until a dose was reached (5 μ g per day) that either fully prevented or cured the lesions in a period of 5 weeks.

The possibility that the infarcts could be the result of a secondary deficiency induced by the limited intake of basal diet was ruled out by the experiments already described elsewhere in this paper. The fact that the same type of lesion was observed with two different PGA inhibitors gave further support to the theory that this abnormal condition of the spleen is one of the manifestations of PGA deficiency in the rat. It seems that a definite concentration of PGA has to be maintained in rat tissues to prevent infarct formation in the spleen.

It is well-known that the spleen is a dispensable organ. Splenectomized healthy animals live their full span of life quite as normally as those with spleens. It is not surprising, therefore, that several rats, not described in this report, fed the basal diet supplemented with a suboptimal level of PGA, lived for several months with severely infarcted spleens. It seems that the development of this lesion is not an immediate cause of death.

In view of the high percentage of animals suffering from infarction during the depletion period, it appears difficult to believe that regeneration did not take place in a substantial number of those animals having normal spleens at the end of the 5-week supplementation period. But direct proof that regeneration takes place is still forthcoming.

SUMMARY

1. Twenty-one-day-old Wistar rats, fed a PGA-deficient ration containing 2% SST as inhibitor, developed marked deficiency symptoms in an average of three to 4 weeks on the ration.

2. Full remission of the depletion symptoms was obtained by administration of 5 μ g of PGA 6 times per week during a period of 5 weeks.

3. Levels of PGA below this amount induced partial remission of some, or all, the symptoms at rates that were roughly proportional to the level of PGA fed to the animals.

4. A characteristic lesion, hitherto undescribed, consisting of infarction of the spleen, was observed in a high percentage of the PGA-deficient animals. No such lesion was found in animals that had received 5 μ g or more of pteroylglutamic acid per day as a dietary supplement during a period of 5 weeks subsequent to depletion.

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REPRODUCTION AND LACTATION STUDIES WITH RATS FED NATURAL AND PURIFIED RATIONS ¹

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INTRODUCTION

Hayward et al. ('34) demonstrated that a corn-soybean-alfalfa ration was adequate for growing and fattening swine. Hayward and associates ('36) found that when soybeans are heated the sulfur-containing amino acids become more available to the experimental animals.

Ross et al. ('44) and Cunha et al. ('44a), using a corn-soybean oil meal-alfalfa ration, had difficulty in reproduction with swine. Few pigs were weaned and symptoms of congenital malformation were found in young pigs from second generation gilts raised on the basal ration. Often the sow died of toxemia during pregnancy. The basal ration consisted of 17.50% soybean oil meal, 76.35% yellow corn, 5.0% alfalfa meal, and 1.15% minerals.

Ross ('43) found that reproduction and lactation in rats were improved when the basal diet was supplemented (at the expense of the corn) with either fish meal, 1:20 liver concentrate, 10% additional alfalfa meal, tankage, dried brewers' yeast, soybean, lecithin or valine. Increasing the calcium and phosphorus level and adding manganese to the diet also

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gave slightly better results. Regularity in estrus cycle was obtained when B₁, B₆, niacin, α -tocopherol and shark liver oil were added to the basal ration.

Cunha ('44) found differences in the growth of rats which he attributed to variations in quality of dietary protein. The reproduction and lactation factor (or factors) was shown to be stored or retained in the rat for the production of three successive litters. The basal ration supplemented with 5% acid-washed casein plus choline gave normal reproduction and lactation, but 5% vitamin-free commercial casein² did not. Supplements to the basal ration of kidney residue plus inositol, or inositol plus choline plus B₆ plus 2% 1:20 liver powder, or 2% solubilized liver extract plus inositol, stimulated reproduction and lactation. The addition of B₆, choline, inositol, biotin, calcium pantothenate and folic acid was found essential for normal growth in rats.

In further studies Spitzer et al. ('46a) showed that this basal ration was inadequate for reproduction and lactation in rats; in this study toxemia symptoms similar to those occurring in swine fed this diet were noted. The inadequacy of the ration produced lactation failure and some reproduction difficulties.

Spitzer ('47) formulated the basal "T" ration, composed of 20% soybean oil meal, 65.84% yellow corn (ground), 5.00% dehydrated alfalfa meal, 2.16% salts (Spitzer and Phillips, '46a), 2.00% Salts IV (Phillips and Hart, '35), and 5.00% corn oil, plus the following per 100 gm of ration: 0.4 gm L(+)-cystine, 0.04 gm α -tocopherol, 0.2 gm halibut liver oil, 0.3 gm inositol, 0.3 gm choline chloride, 5 mg menadione,³ 20 mg ascorbic acid, 30 mg p-aminobenzoic acid, 0.1 mg folic acid, 0.01 mg biotin, 1 mg B₁, 1 mg B₂, 1 mg B₆, 5 mg calcium pantothenate and 5 mg niacin. This fortified basal diet improved the reproduction and lactation performances of female rats by enabling them to wean 32% of their young. The further addition of 5% soybean oil meal at the expense of corn was

² Labco.

³ 2-methyl-1,4-naphthoquinone.

detrimental and resulted in the weaning of only 17% of the young. Bethke et al. ('46) found that supplementation of an all-plant basal diet for laying hens with soybean oil meal resulted in poor hatchability. Whitson et al. ('46) observed a decrease in the hatchability of eggs as the soybean oil meal was increased from 0 to 40% in the diet, despite the apparent adequacy of the dietary factors affecting hatchability. Spitzer ('47) demonstrated that reproduction and lactation were further improved by supplementing the basal "T" ration with either 5% crude casein, 5% fish meal, 0.3% soybean lecithin, 5% 1:20 liver powder, or 10% additional alfalfa meal.

This paper reports further studies on reproduction and lactation in rats fed our basal corn-soybean oil meal-alfalfa diet.

EXPERIMENTAL

Procedure

In this work Sprague-Dawley albino rats and their weanlings weighing over 30 gm at the time of weaning were used. The females were conditioned or standardized carefully as outlined below. The rats were raised to maturity on the stock ration. Prior to mating all the female rats to be used were depleted for three weeks on the basal ration. They were then mated for the first time, at 110-120 days of age. The rats were permitted to bear three litters and were then discarded. Only healthy appearing rats were used for the experiments. The female rats were given a three-week rest period between weaning their young and remating; during this period the animals received the stock ration. A record was kept of the age, the number of litters produced and the strain source (Sprague-Dawley or inbred rats of Sprague-Dawley origin). In each experiment, with the exceptions noted, only rats similar on all three counts were used.

The cages used for mating were large, wire-mesh, false bottom, galvanized steel cages. A maximum of 5 females per cage was allowed during mating. The males used for mating were changed each week. When the females became pregnant

(detected by marked weight gain), they were isolated in smaller galvanized steel cages with galvanized steel bottoms. Clean wood shavings of soft texture and free from sawdust were used for nesting. The wood shavings were changed once a week.

Since early lactation failure occurred in the females fed the basal ration, lactation performance and the weaning of young are best portrayed by the per cent of young weaned. This figure was obtained by dividing the number of young weaned by the number of young born. In order to reduce as much as possible the effect of litter size upon the growth rate of the young, all litters were reduced 48 hours after birth to six young. Despite this precaution, in most cases the litter load was not entirely equilibrated. The young were weaned at 21 days of age. The diets were closely similar in caloric value. Since the object of this study was qualitative and not quantitative in nature, and in view of the inherent difficulties of controlling food intake and food waste by young rats, food consumption data were inaccurate and therefore all rations were fed *ad libitum*. Birth weights were not recorded, since the size of the litter overshadows dietary influence upon the weight of young at birth.

In the preliminary experiments (Maruyama, '48) to test the stimulatory effect of added casein or casein hydrolysates, only slight, if any, stimulatory effect upon reproduction and lactation was obtained. Supplementing basal ration "T" with either crude or purified casein did result in less loss of weight by the suckling mother and somewhat greater gains in body weight of the nursing young. Hydrolysis of the casein by either acid or enzymes apparently destroyed the growth-promoting principle. The addition to the basal "T" ration of the 19 amino acids equivalent to 5% casein resulted in excellent reproduction and lactation performances in preliminary tests with rats; however, the growth factor for the young was not present in adequate amounts, if at all. Thus the preliminary results suggested that the missing lactation

factor in the corn-soybean oil meal-5% alfalfa diet may actually have been low levels of certain of the amino acids.

Experiment I

With these indications from preliminary experiments in mind, the amino acid content of our natural ration was calculated on the basis of figures for the 10 essential amino acids of the foods used here given by Block ('45) and by Block and Mitchell ('46-'47). It was found that lysine and methionine were lower in the basal "T" ration than in the basal "T" plus 5% crude casein. Furthermore, if the requirement of the rat for the individual amino acids was estimated on the basis of the work of Griffith and Farris ('42), then both lysine and methionine were lower in the basal "T" ration than the recommended dietary intake. When basal "T" was supplemented with 5% casein, the amounts of the two critical amino acids were increased to approximately the recommended levels. The effects of supplementing the basal ration with methionine and lysine equivalent to 5% casein, as well as with folic acid, pancreatin, and corn steep liquor additions, were studied. Pancreatin was used in order to minimize the effect of the antitryptic factor if present in the soybean oil meal; it has been reported by Ham et al. ('45) to be in raw soybean meal.

The results are given in table 1, experiment I. The addition to the basal "T" ration of 0.5% L(+)-lysine, 0.3% DL-methionine, or 0.5% L(+)-lysine plus 0.3% DL-methionine gave increased performance, in the order mentioned, in our inbred rats, with normal reproduction and lactation in the case of the last-mentioned supplement. The addition of pancreatin did not have a beneficial effect. The omission of folic acid from the basal "T" ration produced a marked inhibitory effect on reproduction and lactation, and in two of the females resorption occurred. The addition of 5% corn steep liquor had no beneficial effect on lactation as indicated by the per cent of young weaned; however, the growth rate of the young may have been stimulated by this ingredient.

TABLE 1
The effects upon reproduction of various supplements to the corn-soybean meal-alfalfa hay ration

RATION	NO. OF FEMALES	RATS PREGNANT	NO. OF LITTERS	TOTAL NO. OF YOUNG BORN	RATS WEANED ¹	AVERAGE WEANING WEIGHT ² gm	AVERAGE WEIGHT CHANGE OF MOTHER ³ gm	SIGNIFICANCE ⁴
		%		Experiment I	%	gm	gm	
1. Basal "T"	10	100	9	72	46	36.4	+ 27.5	..
2. Basal "T" + 0.5% L(+) -lysine	10	90	9	65	58	37.4	+ 8.9	2.72
3. Basal "T" + 0.3% DL-methionine	8	100	8	58	64	38.5	+ 4.5	5.21 ⁴
4. Basal "T" + 0.5% L(+) -lysine + 0.3% DL-methionine	9	100	9	64	72	35.8	+ 6.4	10.58 ²
5. Basal "T" + 1% pan-creatin	9	100	9	52	48	37.0	+ 11.0	0.185
6. Basal "T", minus folic acid	10	100	8 ^a	41	34	36.0	— 4.0	1.03
7. Basal "T" + 5% corn steep liquor	8	87.5	7	48	42	40.2	+ 23.6	0.04
				Experiment II				
1. Basal "T"	8	87.5	7	56	29	31.7	— 22.7	..
2. Basal "T", minus folic acid	8	100	7 ^a	48	0	1.42
3. Purified ration (with folic acid)	4	100	4	27	59	42.8	+ 32.5	..
4. Purified ration (minus folic acid)	5	100	3 ^a	19	10	38.0	+ 29.0	9.20 ²

¹ Per cent weaned calculated on basis of the number of young born.

² The growth and weight change data of the mother rats are of doubtful significance because of the unequal "litterload."

³ Chi square significance data were calculated from the "per cent weaned" column and the values shown are compared with the performance record of group 1, basal "T."

⁴ P value exceeds 5% level.

⁵ P value exceeds 1% level.

⁶ Resorption occurred in the remainder of the females.

If the per cent weaned was calculated on the basis of the number of live young at 48 hours it appeared that the addition of amino acids improved lactation, since 73% of the young alive at that time were weaned from mothers fed the basal "T" ration as against 83%, 86%, and 96% weaned from mothers fed the basal "T" ration supplemented with lysine, methionine or lysine-methionine, respectively.

Experiment II

The study of the effect of folic acid on reproduction and lactation was repeated in a second experiment. Many workers believe that folic acid is synthesized in limited amounts by the intestinal flora of the rat. They believe also, that when the carbohydrate of the purified ration is sucrose, the synthesis of folic acid as well as other "B complex" vitamins is reduced to a minimum. Therefore, a purified ration developed by Sporn et al. ('47) was used, with a slight modification for this experiment. It consisted of sucrose 73%, casein⁴ 18%, corn oil 5% and Salts IV (Phillips and Hart, '35) 4%, plus the following vitamins in milligrams per 100 gm of ration: B₁, 0.3; B₂, 0.3; B₆, 0.2; niacin, 2; calcium pantothenate, 2; folic acid, 0.025; biotin, 0.01; inositol, 0.01; choline chloride, 100; and *p*-aminobenzoic acid, 25. Instead of feeding 10 mg of α -tocopherol per rat at the beginning of the experiment and two drops of one to 4 dilution of oleum percomorphum per week, the vitamins E, A and D were incorporated into the ration and folic acid was increased from 25 μ g to 100 μ g per 100 gm of the ration. Alcohol-extracted casein was used in place of the commercial preparation.⁵

The results are given in table 1, experiment II. The Sprague-Dawley rats on basal "T" weaned 29% of their young, while those on basal "T" minus the folic acid weaned 0%. One resorption occurred. Thus the essentiality of folic acid was again demonstrated. When the purified ration with

⁴ SMACO.

⁵ See footnote 4.

folic acid was used, 59% of the young were weaned, and only 10% when the folic acid was omitted from the ration. Resorption occurred in two out of the 5 rats used.

It seems evident from these data that folic acid is a factor essential for normal reproduction and lactation in the rat when either a purified or natural ration is employed.

DISCUSSION

Spitzer ('47) believed an unknown factor or factors necessary for reproduction and lactation in the rat to be present in casein. Johnson et al. ('42) have shown the presence in casein of a chick growth factor. Woolley and Collyer ('46) demonstrated that strepogenin is essential for optimal growth in mice. Scott et al. ('47) showed that the strepogenin found in 2.2% crude casein or in 10% yeast produced a two-fold gain in chick growth when the source of protein in the purified diet was 20% egg albumin (autoclaved) and 10% gelatin.

Stimulation of reproduction and lactation equivalent to that obtained by the addition of 5% crude casein to the basal "T" ration formulated by Spitzer ('47) was obtained when the 19 amino acids equivalent to 5% casein were substituted for the casein. Addition of 0.5% L(+)-lysine and 0.3% DL-methionine to the basal "T" ration brought about excellent reproduction and lactation in the rat; thus it appears that such supplementation provides additional stimulation for these functions. The requirement of extra methionine for the adequacy of soybean oil meal protein has been demonstrated by Almquist et al. ('42). Russell et al. ('46) showed that methionine enhanced growth in rats fed soybean oil meal rations.

In our studies folic acid, among other factors, has been shown to play an important role in the reproduction and lactation of the rat. Possibly the requirement for folic acid increased during the gestation period and the amount synthesized in the digestive tract plus the small amount in the constituents of the diet were not sufficient to promote normal gestation and parturition. Hence, resorption occurred fre-

quently. Recently, Lillie and Briggs ('47) determined by chick assay the folic acid activity in common feedstuffs and reported alfalfa meal to contain 11.3 μg per gram and liver and yeast 50 μg per gram. This may explain in part the stimulation obtained by supplementing alfalfa meal, liver or yeast to the basal diet by Ross ('43), Cunha ('44), and Spitzer ('47). Cerecedo et al. ('44, '47) showed the beneficial effect of folic acid on lactation in mice, using a highly purified ration. Fenton and Cowgill ('47) found that some stimulation for reproduction and lactation in their highly inbred strain of mice resulted when folic acid was added to a purified diet. Bowland et al. ('48) reported stimulation from folic acid for reproduction and lactation in rats but not for growth.

Due to the unequal number of young that the mother rats nursed to weaning, the data on the average weaning weight of the young and the weight changes in the mother rats during the gestation and lactation period can be considered only as suggestive but not significant in determining the adequacy of the diet in these experiments, a point stressed by Nelson and Evans ('47).

Efforts to supplement adequately a yellow corn-soybean oil meal-5% alfalfa ration in order to stimulate reproduction and lactation in the rat have shown that choline, B₆, inositol (Ross, '43; Cunha, '44) and biotin (Spitzer, '46b) improve the ration as regards these important functions. Further improvement has now been attained by additional supplements of 0.5% L(+)-lysine and 0.3% DL-methionine and folic acid.

SUMMARY

1. Standardization of the experimental animal was found to be essential for reproduction and lactation studies.

2. Supplementing the basal "T" ration with 0.5% L(+)-lysine and 0.3% DL-methionine led to excellent reproduction and lactation in rats. The weight of the young weaned on this ration was less than that of young weaned on the casein-supplemented ration.

3. Supplemental folic acid appeared to be essential for normal reproduction and lactation in our female rats fed the corn-soybean-5% alfalfa ration or a purified ration.

4. The addition of 1% pancreatin or 5% corn steep liquor to the ration did not have any stimulative effect on reproduction or lactation, but 5% corn steep liquor did stimulate growth in the suckling young.

5. From these studies it is evident that in addition to other factors demonstrated to be helpful in reproduction and lactation — i.e., choline, B₆, inositol, and biotin — the amino acids L(+)-lysine and DL-methionine, and folic acid, are essential for good reproduction and lactation in female rats fed basal ration "T," which is essentially a corn-soybean oil meal-5% alfalfa ration.

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THE LACTATION RESPONSE AS LIMITED BY FEEDS PRODUCED UNDER TWO SYSTEMS OF SOIL FERTILIZATION

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ONE FIGURE

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Observations of certain workers indicate that varying levels of soil fertility have affected through the medium of plants the nutrition and milk production of dairy cattle (Pipers, '09; Green, '25). In other studies the weight gains of animals have been influenced (Theiler et al., '20, '24; Crampton, '34; Albrecht and Smith, '41; Webb et al., '46; Turner et al., '46). Improved palatability (Nevens, '41; Archibald et al., '43) and an increased apparent digestibility of certain forages (Nehring, '38; Eheart and Pratt, '42) have been attributed to soil fertilization; in other work the biological value of proteins in barley (Nehring and Schramm, '40) was not improved by such means. Other investigators report improved animal reproductivity, health and longevity resulting from soil fertilization (Hunt, '27; Tallarico, '31; Scheunert et al., '34).

The effects of varied levels of soil fertility upon animals have been investigated largely through the feeding of grasses or through range grazing studies. Because of the relatively meager evidence relating to such levels as they affect cereal grains and legumes, and to their ultimate influence upon the

¹The data of this paper were taken from a thesis submitted by this author to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

nutrition of animals, further study of the general problem seemed justified.

The object of this investigation was to study the lactation response in the rabbit as it is influenced by the nutrient content of feeds produced on Cisne silt loam fertilized with phosphorus and potassium and with or without calcium.

The cooperation of the Department of Agronomy of the University of Illinois made this study possible by supplying the cereal grain and legume roughages fed, which were grown on the Soil Experiment Field located at Newton, Illinois. This field was established in 1912. Since the time of its establishment accurate records have been kept of the fertilizers applied and their effects upon the productivity of the soil as shown by crop yields (Bauer et al., '45).

EXPERIMENTAL

The effects of two series of fertilizer applications upon the nutritive value of wheat grain, lespedeza hay, and soybean hay were studied through the use of paired feeding trials with lactating rabbits and their nursing young. The fertilizer series and the amounts of each fertilizer applied per acre since the field experiments were started were as follows: Series 1, raw rock phosphate, 4 tons; kainit, 2.25 tons plus 1,000 pounds of muriate of potash. Series 2 was similar to series 1 in that 4 tons of raw rock phosphate, 2.25 tons of kainit, and 1,000 pounds of muriate of potash were used, but differed from series 1 in that 12 tons of limestone were also applied. For the sake of clarity and brevity, series 1 will be referred to as the PK and series 2 as the PK + Ca treatment.

Trial 1: Lespedeza hay and wheat studies

Equal parts of ground wheat grain and ground Korean lespedeza hay (1945 crops), produced either on the PK or the PK + Ca series, and 1% sodium chloride (C.P.) were used in making up two rations. The first was known as the PK ration, the second as the PK + Ca ration. The lespedeza

produced under each soil treatment was cut on the same day, while the crop was in the early to medium bloom stage. The hay was cured under good hay-making conditions and stored with a minimum of leaf loss. Before grinding the lespedeza all of the foreign matter, chiefly weeds, was removed. The lespedeza contents of the forage as harvested from the PK and PK + Ca-treated plots were 33.3 and 71.5%, respectively.

The total nitrogen content of the lespedeza hay from the PK-treated plots was 1.96% and for the hay from the PK + Ca soil treatment, 2.36%. The two lots of wheat contained 1.53% and 1.94% nitrogen, respectively, for the PK and the PK + Ca treatments.

The percentages of dry matter, total nitrogen, and ash for the two rations as fed were (in the order named) 89.3, 1.74 and 4.6 in the PK ration and 89.9, 2.16 and 4.4 in the PK + Ca ration.

The rabbit was used as the experimental animal. Previous investigations (Crampton et al., '40; Smith and Albrecht, '42) with the rabbit have shown it to be similar to cattle or sheep in its ability to utilize feeds.

Six pairs of litter-mate New Zealand white females and two related males were used. The female of each pair was identified by a number followed by the letter "—O" if fed the PK ration, or "—W" if fed the PK + Ca ration.

Throughout the study, the paired feeding technique was used to equalize food intakes (Mitchell and Beadles, '30). After a three-week preliminary feeding period in which the experimental rations were fed, the animals were mated. Each male was mated to two females daily with only one service per female being permitted. This use of the males was not believed to be excessive (Jones and Hays, '18).

Though the females were mated at essentially the same time, because of the variability in the length of the gestation periods age in the young was calculated in days from the probable time of conception. In most instances the young were born by the end of the 31st day after conception. As soon as possible after birth the young were counted and

weighed. Usually, by the third day the litters were standardized to 6 young or less.

The feeders used were mounted high enough to prevent the young from gaining access to the rations fed. Thus the milk of their mothers was the only source of nutrients for the young.

Daily weights of litters were taken until the 46th day from the probable date of conception, at which time the litters were sacrificed. The animals of a litter were weighed individually and were measured for body length. After removal of gastrointestinal contents the carcasses were weighed, frozen and later ground for analysis.

The individual carcasses were analyzed for dry matter, total nitrogen and ash. In all, 46 carcasses were analyzed, comprising an equal number from each of the two groups compared. Because the entire litter of doe 8-O (PK) died on the 35th day after conception, the observations taken for doe 8-W (PK + Ca) and her litter have been omitted from the reported data.

Dry matter was determined by holding 1-2 gm samples of ground carcass at 100°C. for 4 hours. Total nitrogen was determined by the official Kjeldahl method (A.O.A.C., '40). Samples for ashing were placed in a muffle furnace. The temperature was slowly raised to 575-600°C. and held for 6 hours.

Trials 2 and 3: Soybean hay and wheat studies

Trials 2 and 3 differed from trial 1 in that excellent quality soybean hay was used to replace the lespedeza hay in the rations fed. The soybeans were grown under the same conditions as was the lespedeza used in the first trial.

The total nitrogen in the wheat (1946 crop) from the PK-treated soil was 1.79%, compared with 2.01% for that produced on the PK + Ca-treated plots. The soybean hay from the PK-treated soil contained 2.39% total nitrogen in contrast to 3.02% for hay from the PK + Ca-treated soil.

The ration used in trials 2 and 3 contained the same proportions of ground wheat, ground soybean hay, and sodium

chloride as were used in trial 1. The percentages of dry matter and total nitrogen in the rations as fed were 88.7 and 2.06 respectively, for ration PK, and 89.1 and 2.49 respectively for the PK + Ca ration.

TABLE 1
Weights of does and their food intakes

DOE NUMBER	DOE WEIGHTS AT TIME OF:			FEED CONSUMED DURING:	
	Mating	Parturition	Litter sacrifice	Gestation	Lactation
	kg	kg	kg	kg	kg
Trial 1: Wheat and lespedeza hay study					
1-O ¹	3.9	3.8	3.5	3.1	2.0
1-W ²	3.9	3.8	3.6	3.2	2.0
2-O ¹	4.0	3.9	3.7	3.7	2.2
2-W ²	4.3	3.9	3.8	3.6	2.3
3-O ¹	4.3	4.1	3.9	3.4	1.8
3-W ²	3.7	3.5	3.5	3.4	1.8
6-O ¹	3.9	4.0	3.7	4.1	2.6
6-W ²	4.3	4.3	3.9	4.1	2.6
7-O ¹	3.9	3.9	3.4	3.6	2.6
7-W ²	3.6	3.8	3.4	3.6	2.6
Trial 2: Wheat and soybean hay study					
1O2 ²	4.9	4.5	4.5	2.3	2.6
1W2 ¹	4.4	4.1	4.0	2.3	2.7
5-2 ²	3.9	3.9	4.2	4.8	2.8 ³
8W2 ¹	3.4	3.6	3.7	3.2	2.8 ³
Trial 3: Wheat and soybean hay study					
5-3 ²	3.7	3.7	3.8	3.2	2.7 ³
2W3 ¹	3.7	3.5	3.7	4.2	2.7 ³
Average ¹	3.9	3.8	3.7	3.4	2.4
Average ²	4.0	3.9	3.8	3.5	2.4

¹ Ration from PK-treated soil.

² Ration from PK + Ca-treated soil.

³ Paired only during lactation.

Approximately 6 months after the completion of the wheat and lespedeza feeding studies the same females were again placed on experiment. In these studies the females were reversed with reference to the soil treatments as affecting the rations.

RESULTS

The PK + Ca ration that contained lespedeza appeared to be slightly more palatable than the PK ration. This was indicated by the quantity of feed that remained in the feeders

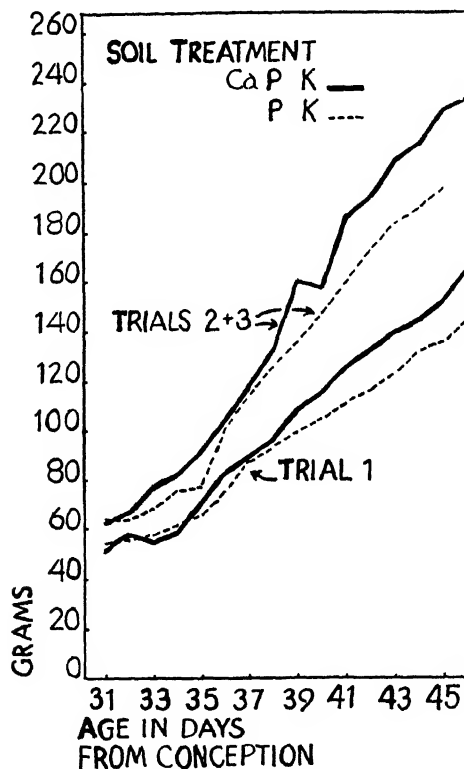


Fig. 1 The average daily weights of nursing rabbits whose mothers, in trial 1, were fed lespedeza hay and wheat and in trials 2 and 3, soybean hay and wheat. All feeds were grown on a soil that had been treated with two different combinations of fertilizers.

each day. Four of the 5 does receiving ration PK, which contained lespedeza, limited the feed intakes of their pair mates. When the soybean hay and wheat rations were fed, the tendency to limit the food intake of pair mates was about evenly divided between the two soil treatments.

A study of the average weights of the lactating females as shown in table 1 indicates that the average total weight losses while on the two rations were approximately equal. The average losses were 0.24 and 0.21 kg respectively for the PK and the PK + Ca treatments. These losses occurred from the time the females were mated until the time the young were sacrificed, 46 days later.

TABLE 2

Average body measurements and carcass composition of litters in trial 1

LITTER NUMBER	NUMBER OF YOUNG	BODY MEASUREMENTS			CARCASS COMPOSITION		
		Length	Full wt.	Empty wt.	Dry matter	Total nitrogen	Ash
		<i>cm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
1-W ¹	6	18.6	135	124	29.0	3.3	4.2
1-O ²	6	17.2	110	102	25.2	2.8	3.6
2-W	3	20.0	193	175	40.9	4.9	6.3
2-O	2 ³	18.3	163	141	33.2	3.9	5.0
3-W	4	19.9	177	156	35.2	4.0	5.0
3-O	4	18.3	143	126	28.4	3.4	4.0
6-W	5	21.3	219	198	54.7	5.3	5.9
6-O	5	19.9	189	173	49.7	4.5	5.8
7-W	5 ⁴	20.1	170	154	40.2	4.4	5.6
7-O	6	19.0	153	146	39.1	4.2	5.2
-W	23	19.9	176	159	39.6	4.3	5.4
-O	23	18.5	149	136	35.3	3.7	4.7

¹“-W” = PK + Ca ration (wheat and lespedeza).

²“-O” = PK ration (wheat and lespedeza).

³One animal died 4 days before the litter was sacrificed.

⁴One animal died two days before the litter was sacrificed.

The pairs of mating totaled 17. Of the 17 females that received the PK rations, 14 bore litters averaging 6.43 young per litter, with three of the females failing to conceive. Twelve of the 17 females receiving rations PK + Ca produced an average of 7.75 young per litter, with the 5 remaining females failing to have litters. Birth weights of the individual animals in any litter were inversely related to the number of animals in the litter.

The average daily weights of the litters of nursing dams receiving the PK + Ca rations were larger than those of the litters of their paired litter mates. The differences in the average daily weights of litters were apparent after the 38th day from conception, as is shown in figure 1.

As is indicated in table 2, the average body length for the 23 young in trial 1, whose dam had been fed ration PK, was 18.5 cm as compared with an average body length of 19.9 cm

TABLE 3
Average body measurements of litters in trials 2 and 3

RATION	LITTER NUMBER	NUMBER OF ANIMALS	BODY MEASUREMENTS:		
			Length	Full wt.	Empty wt.
			<i>cm</i>	<i>gm</i>	<i>gm</i>
Trial 2 ¹					
PK	1W2	3	20.8	230	209
PK + Ca	1O2	3	21.4	251	218
PK	8W2	4 ²	20.2	219	186
PK + Ca	5-2	5	21.1	210	181
Trial 3 ¹					
PK	2W3	3 ³	19.2	205	182
PK + Ca	5-3	3	23.1	289	257
PK		10	20.0	218	191
PK + Ca		11	21.7	242	211

¹ Wheat and soybean hay studies.

² One animal died two days before the litter was sacrificed.

³ One animal in this litter was quite small, weighing only 92 gm at the time of sacrifice.

for those whose dams had been fed ration PK + Ca. Although a smaller number of young survived in trials 2 and 3, their body lengths for the PK and PK + Ca rations were 20.0 and 21.7 cm respectively.

The average weight of stomach and intestinal contents of the 23 PK young in trial 1 was 12.6 gm, as compared to 16.8 gm for the 23 young reared from does on PK + Ca rations. The same trend appears in trials 2 and 3 where the gastro-

intestinal contents were 27 and 31 gm, respectively, for the PK and PK + Ca treatments.

The average quantities of dry matter, total nitrogen, and ash deposited in the carcasses, as shown in table 2, were 35.36, 3.86 and 4.79 gm respectively for those animals affected by the PK treatment. The percentages of these same components in the carcasses were: dry matter, 25.52; total nitrogen, 2.78; and ash, 3.51. The quantities of these deposition products were in direct contrast to those for the PK + Ca animals. In this group the deposited dry matter, total nitrogen and

TABLE 4

Analysis of the variance between the PK and PK + Ca treatments as affecting carcass measurements and composition of animals in trial 1

CARCASS FACTORS ANALYZED	F	COEFFICIENT OF VARIATION
		%
Body length	30.3 ¹	4.5
Gastrointestinal contents	12.9 ¹	26.6
Body weight (empty)	19.4 ¹	11.6
Dry matter deposited	8.0 ¹	13.6
Nitrogen deposited	26.5 ¹	19.0
Ash deposited	10.5 ¹	11.9
Dry matter (%) ²	13.9 ¹	3.2
Nitrogen (%)	.0025	7.9
Ash (%)	2.9	6.6

¹ Significant at the 1% level of probability.

² Significantly greater in the young of the PK-nourished mothers.

ash averaged 39.66, 4.42 and 5.36 gm, respectively. Percentage values for these same components in the order named were 24.65, 2.75 and 3.39.

An analysis of the variance in the carcass observations in the first trial is summarized in table 4. The variance between treatments with respect to body lengths, gastrointestinal contents, empty carcass weights, quantity of dry matter, total nitrogen, and ash deposited was in every instance sufficiently greater than the error variance to be highly significant at the 1% level of probability. The variance between treatments

with respect to the percentage of total nitrogen and ash was only slightly greater than the error variance and was therefore not significant.

Although the differences between the values for average body lengths, gastrointestinal contents and empty carcass weights of the young produced in trials 2 and 3 showed the same trends as did those in trial 1, the number of degrees of freedom was not sufficiently large to establish the significance of the differences observed.

DISCUSSION

The causes of the wide differences in the nutritive values of the feeds grown under the two systems of soil treatment are not apparent from the data presented in this paper. It is possible that a simple correction of the protein disparity might suffice to equalize the two rations. On the other hand, it seems probable that the differences are more deep-seated than a mere difference in the nitrogen contents of the rations. Did the two systems of soil treatment cause differences in the physical as well as the chemical structure of the plant tissues? Did the addition of calcium to one series of plots enable the plants grown thereon to absorb more of some of the trace minerals? In order to determine the underlying causes of the differences which may be produced in animals by the two systems of soil fertilization, further careful research is needed. Such research is now in progress.

SUMMARY

Differences in the nutritive values of lespedeza hay, soybean hay, and wheat grain were carefully appraised through the lactation responses of rabbits. The use of doe litter mates and the paired feeding technique made such an evaluation possible.

Data are reported for 17 pairs of pair-fed, mated, doe rabbits whose rations were made up of feeds produced on a single soil type; and for 8 pairs of litters produced by these does, to an age of 46 days from conception. One ration was made

up of feeds grown on PK-fertilized soil; the other consisted of feeds grown on a similar soil fertilized with PK + Ca.

The PK + Ca rations were found to be superior to the PK rations in the following respects:

1. In trial 1 they appeared to be more palatable. No noticeable differences in palatability were observed in trials 2 and 3 when soybean hay replaced lespedeza hay.

2. The average litter size showed some tendency to be larger.

3. Average daily weights of the young were greater after reaching an age of 38 days from conception.

4. An analysis of the variance of the trial 1 data showed that body length, gastrointestinal contents, and the quantity of dry matter, total nitrogen, and ash deposited in the young fed solely on the milk of mothers fed the PK + Ca ration were significantly greater than in their PK-nourished pair mates.

Differences in the percentages of carcass nitrogen and ash were not significant.

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THE EFFECT OF PREVIOUS DIET ON THE ABILITY OF ANIMALS TO DO WORK DURING SUBSEQUENT FASTING¹

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ONE FIGURE

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The present investigation was begun in order to determine the effect of diet on the ability of animals to withstand adverse conditions during subsequent fasting. Previous work has indicated that the metabolism of the peripheral tissues of animals is affected by the prevailing major energy component of the diet. This effect tends to persist during subsequent fasting (Guest, '41; MacKay, Carne, Wick and Visscher, '41; Samuels, Reinecke and Ball, '42; Roberts, Samuels and Reinecke, '43). Experiments were therefore set up in which the daily caloric intake of all groups of animals was maintained at the same level by forced feeding but was supplied largely by either carbohydrate, fat, or protein. In all groups sufficient protein was fed to maintain an adequate amino acid supply. The animals were placed in metabolism cages which also recorded activity. Voluntary activity and

¹The research reported in this paper has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained herein are those of the authors; they are not to be construed as necessarily reflecting the views or endorsement of the War Department.

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nitrogen balances were determined during the feeding period. Fasting was then begun, and at intervals the amount of exercise required to produce complete exhaustion was determined. The animals were fasted until they died of either exhaustion or starvation.

METHODS

In the present experiments, male rats obtained from Sprague-Dawley, Inc., were placed in combined activity and metabolism cages when they weighed 150–175 gm. The cages were designed by Dr. Reinecke. Each consists of a cylindrical revolving cage of low inertia which drives a counting mechanism that adds every inch of movement in either direction around the axis. The cage in turn can be driven by sprocket and chain from a variable speed motor. To prevent the animal from riding the cage, a baffle is introduced at the top. Below the cage are a funnel and a collecting mechanism for recovering urine and feces separately. All of the parts which come in contact with the animal or its urine and feces are built of stainless steel, thus reducing the possibility of contamination with metals and the consequent catalysis of the destruction of certain organic molecules.

After being placed in the cages, the rats were fed a stock diet of calf meal *ad libitum* until they weighed 250 gm or more. During this time records of voluntary activity were kept. The rats were then placed on one of the diets shown in table 1. These were fed by stomach tube in equicaloric amounts, the usual intake being 52 calories per day, which was divided into two or three feedings. Forced feeding eliminated the variability in rate of food intake which has been shown by Tepperman, Brobeck and Long ('43) to have an influence on the type of metabolism. Records of voluntary activity were also kept during the periods on the experimental diets.

At the end of 4 weeks on the experimental diets the animals were fasted. Exhaustion tests were run from one to 4 days later and thereafter at intervals of one week until the animals

died. Voluntary activity was recorded during the intervals between exhaustion tests.

In two experiments blood sugar samples were taken during the feeding period. In all experiments blood sugar levels were determined 12 hours after beginning the fast and before and after each exercise period; sometimes at more frequent

TABLE 1
Diets used in stomach tube feeding

	HIGH FAT	HIGH CARBOHYDRATE	HIGH PROTEIN
Lactalbumin, Labco (gm)	30	30	263.25
Gelatin, U.S.P. (gm)	30	30	30
Corn oil (gm)	105.8	3	3
Dextrin (gm)	0	233.25	0
Salt mixture, U.S.P., no. 1 (gm)	12.0	12.0	12.0
Oleum percomorphum, reinforced ¹ (drops)	12.0	12.0	12.0
Vitamin concentrate ² (gm)	6.0	6.0	6.0
Water to make (ml)	900	900	900
Calories per ml	1.30	1.31	1.30
Caloric distribution: (% of total calories)			
Protein	19.5	19.5	92.4
Fat	80	2.7	2.7
Carbohydrate	0.5	77.8	4.9

¹ Mead Johnson and Company.

² Lederle's Lederplex. According to label, 6 gm contained:

Thiamine hydrochloride	24 mg	Calcium pantothenate	36 mg
Riboflavin	24 mg	Choline	240 mg
Nicotinamide	120 mg	Inositol	120 mg
Pyridoxine	2.4 mg	Folic acid	2.4 mg

Plus other water-soluble extractives from 96 gm liver.

intervals. The sugar was determined by the Reinecke modification of the Folin-Malmros method (Reinecke, '42).

Urine and feces were collected twice a week during the feeding period. A small amount of sulfuric acid and a layer of toluene were used as preservatives. During fasting the urine and feces were saved, up to the time of forced exercise. They

were then collected separately for the period of exercise and the succeeding 12 hours. This procedure of separating the samples during voluntary exercise from those of the periods of forced exercise was continued until the death of the rat. The feces and urine were each analyzed for nitrogen by the Kjeldahl method.

RESULTS

In all tables mean values are given for each group. In most instances the standard error of the mean is also given. Student's "t" test as applied to small groups has been used to determine the probability of the significance of differences observed.

Activity during the feeding period

Table 2 gives the average voluntary activities of the experimental animals, both during the feeding periods and during fasting.

The outstanding point shown is the change which occurred when a diet high in any one food factor was substituted for a mixed diet. The greater activity did not seem to be due to some special ingredient of the stock calf meal mixture, since the rate was maintained when, in experiment 2, the rats were transferred to a force-fed diet consisting of a mixture of equal parts of the high carbohydrate and high fat diets. Later, when these rats were transferred to either the high carbohydrate, high fat or high protein diet, the voluntary activity dropped to approximately one-half the rate on the force-fed mixed diet, a drop similar to that seen when the rats were transferred directly to the specific diets from the calf meal. This change was highly significant by Student's "t" test. On the other hand, there was no difference of any magnitude between the three diets high in one factor as a source of energy. Apparently a rat desires physical activity most when it is utilizing all three foodstuffs.

TABLE 2
Voluntary activity of rats during various types of feeding and periods of fasting

DIET	RATS FED	REVOLUTIONS PER DAY										Fasting 14th-last days
		Calf meal ad libitum	Forced-fed mixed diet	Forced-fed spec. diet	Fasting 1st 4 days	Rats	Rev.	Rats	Fasting 7th-11th days	Rev.	Rats	
Carbohydrate Fat Protein	8	618	Diet not fed	Experiment 1	8	169						
	8	543	Diet not fed	227	8	318					Not measured	
	3	500	Diet not fed	306	3	135					Not measured	
Carbohydrate Fat Protein	7	540	608	Experiment 2	6	366	5	238	5	639		
	6	626	545	308	6	319	5	89	4	154		
	6	600	599	366	5	372						
Carbohydrate Fat Protein	5	632	Diet not fed	Experiment 3	4	312	3	355	3	671		
	4	566	Diet not fed	382	4	110	4	34	3	532		
	5	749	Diet not fed	222	1	63	1	530				
Carbohydrate Fat Protein	7		Diet not fed	Experiment 4								
	5		Diet not fed	359							Not measured	
	6		Diet not fed	225							Not measured	
Carbohydrate Fat Protein	20	598 ± 98		Composite:	18	257 ± 45	8	253 ± 58	8	643 ± 120		
	18	569 ± 82		308 ± 33	18	272 ± 39	9	65 ± 25	7	192 ± 30		
	13	642 ± 130		285 ± 32	9	284 ± 90						
				250 ± 23								
	16		602 ± 133	All force-fed mixed diet								

*Spontaneous activity, forced activity and survival
during fasting*

In table 3 it will be seen that there is a highly significant difference between mean periods of survival for the rats on the different diets. This was in spite of an equicaloric intake during the feeding period. The rats on the high fat diet survived the longest, 18.3 days. The carbohydrate-fed

TABLE 3

*Exercise causing exhaustion of adult male rats, nitrogen excreted and survival
while fasting with periodic exercise*

	CARBOHYDRATE		FAT		PROTEIN	
	Rats	Category of interest	Rats	Category of interest	Rats	Category of interest
Exercise, ¹ 2-4 days fasting (Revolutions)	16	4040 \pm 411	12	6730 \pm 915	7	3600 \pm 262
Exercise, ¹ 9-11 days fasting (Revolutions)	14	8660 \pm 1017	13	8800 \pm 1020	4	7220 \pm 500
Exercise, ¹ 16-18 days fasting (Revolutions)	3	8650	7	7960 \pm 738	0	
Total forced exercise fasting (Revolutions)	14	13800 \pm 1630	12	19700 \pm 1780	9	6465 \pm 1650
Nitrogen excreted per unit work (mg/1000 rev.)	19	.0465 \pm .0058	22	.0342 \pm .0044	11	.0521 \pm .0095
Fasting survival (days)	13	15.5 \pm 0.46	12	18.3 \pm 0.44	9	10.2 \pm 1.68

¹ Rate of exercise was 7 r.p.m. or 20 ft. per min.

animals were next, with an average survival of 15.5 days. This was significantly shorter than that of the fat-fed rats (P less than 0.01) and definitely longer than that of the animals fed protein (P less than 0.01).

When the rats were forced to exercise to exhaustion at some time during the first 5 days, those receiving the high

fat diet ran significantly longer than the carbohydrate-fed group, the mean difference being 2690 revolutions (6730 vs. 4040). This was also significantly longer than the average for the protein-fed rats (6730 vs. 3600). In the second and third exercise periods no significant difference existed. The number of animals surviving for repeated exhaustion trials dropped markedly in the case of the protein-fed group by the time of the second test, and in the case of the carbohydrate-fed group by the third test. There was therefore some selective action. When the mean total forced activity was determined (sum of all exercise tests through which the animal survived), there were significant differences among all three groups, the fat-fed rats being best, those previously on a carbohydrate diet next, and the high protein-fed animals poorest.

One of the interesting observations was that the time required for exhaustion of the rats on any of the diets increased when the second test was run. The change was not so great among the fat-fed animals as in the carbohydrate-fed group. It was impossible to make any quantitative comparisons between the protein-fed rats and the others on the basis of the second exercise period, since so few animals previously on the high protein diet survived long enough. This increased time for exhaustion did not seem to be the result of a simple learning process, since animals exercised immediately at the end of the feeding period (experiment 3) did not show an increase on their next fasting test.

Not only was there an increase in the amount of work required for exhaustion as the fast lengthened, but terminally there was also an increase in the voluntary activity. This was not an immediate effect of inanition, however, since the voluntary activity rates during the first 4 days of fasting did not differ significantly from those for animals on the diets. In fact, the voluntary activity of the rats previously fed a high fat diet gradually decreased, and reached a low point between the 7th and 11th days. As death approached, however, these rats also showed a spurt in voluntary activity.

While the rats previously fed the high fat diet were able to do significantly more work before exhaustion than either of the other groups, their voluntary activity was less. As a consequence, the total activity of the fat-fed rats during fasting, although averaging slightly more, did not differ significantly from that of the carbohydrate-fed group. Both differed significantly from the protein-fed rats because of the high incidence of early deaths among the latter.

Changes in blood sugar during fasting and exercise

The changes in blood sugar levels during fasting are shown in figure 1. Values after forced activity are not included. During the first 6 hours after feeding the glucose level of the

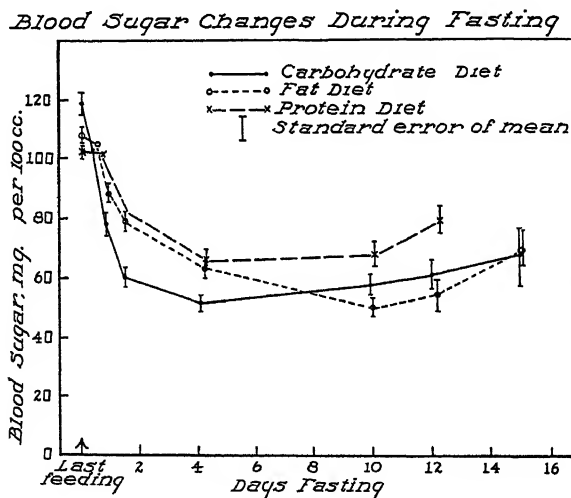


Figure 1

carbohydrate-fed rats was significantly higher than that of the other two groups. It also fell more rapidly in this group, however, so that 8–12 hours after feeding there was no significant difference. By 16 hours after feeding the order had been reversed, the carbohydrate-fed rats having significantly lower blood sugar levels. This difference was still

12 mg per 100 ml at the end of 4 days of fasting, and was highly significant statistically.

By the 10th day, however, the difference had disappeared. In fact, the carbohydrate and protein groups had now apparently higher blood sugar levels than the fat-fed animals. This was due to the fact that more of the animals in the first two groups were approaching death. During the 48 hours preceding death, the blood sugar level rose in all animals. This was also the period of increased voluntary activity; probably the two phenomena are causally related, perhaps via the adrenals.

TABLE 4
Blood sugar changes during forced exercise

	CARBOHYDRATE	FAT	PROTEIN
	<i>mg/100 ml blood</i>		
First exercise period:			
Blood sugar before	55.5 ± 2.8	62.4 ± 2.8	66.2 ± 5.4
Blood sugar at exhaustion	58.9 ± 5.4	88.9 ± 6.3	73.0 ± 8.9
Difference	+ 3.4 ± 6.0	+ 26.5 ± 6.2	+ 6.8 ± 7.1
Second exercise period:			
Blood sugar before	59.9 ± 3.6	57.7 ± 5.7	69.5 ± 6.6
Blood sugar at exhaustion	58.6 ± 4.1	76.6 ± 8.6	72.2 ± 14.2
Difference	- 1.3 ± 5.5	+ 18.9 ± 9.2	+ 2.7 ± 17.2

There were marked differences in the effects of exercise on the blood sugar levels (table 4). When samples were taken at the beginning of forced exercise and at exhaustion, the rats originally fed carbohydrate showed small and irregular differences among themselves. The mean change was a slight increase of 3.4 mg per 100 ml. In the protein-fed group the change was similar. On the other hand, the rats previously fed a high fat diet, with rare exceptions, showed a considerable increase in the blood sugar level during exercise. The average increase was 26.5 mg per 100 ml. Apparently there was a marked difference between the two groups of animals with respect to the balance between glucose production and utilization.

Nitrogen excretion during feeding, fasting and exercise

Nitrogen excretion was measured during the last 5 days on the special diets. As shown in table 5, the nitrogen excretion bore a direct relation to the nitrogen intake and in all groups showed a slightly positive balance consistent with the increasing size of the animals.

During fasting, however, there were significant differences in excretion due to the diets previously fed. In the first 4 days the rats previously fed protein excreted significantly more nitrogen and the fat-fed rats significantly less than

TABLE 5

Nitrogen excretion on special diets and during subsequent fasting with voluntary activity

DIET	ON DIET	1ST FASTING PERIOD (0-4th days)	2ND FASTING PERIOD (6th-11th days)	3RD FASTING PERIOD (13 day-death)
<i>gm N per rat per day</i>				
High carbohydrate	0.3375 \pm .0053	0.1770 \pm .0068	0.1318 \pm .0111	0.2286 \pm .0131
High fat	0.3165 \pm .0080	0.1104 \pm .0057	0.1004 \pm .0094	0.1407 \pm .0272
High protein	1.369 \pm .038	0.2547 \pm .0314	0.1427 \pm .0392	

those of the carbohydrate-fed group. The nitrogen excretion of the carbohydrate-fed group continued to drop, while that of the fat-fed group tended to level off; the difference between them therefore decreased. Terminally there was some increase in the nitrogen output of all animals, the so-called "premortal rise."

Not only was there a significant difference in the nitrogen excretion during fasting associated with voluntary activity, but the fat-fed rats also excreted less nitrogen during the exercise periods. As may be seen in table 3, the nitrogen output per unit of work was definitely lower for these animals. They must therefore have been burning more non-protein com-

pounds. These results agree with the hypothesis that for some time after feeding has ceased the tissues of the carbohydrate-fed rats tend to use glucose more readily for energy, and fat less readily, than do those of the fat-fed rats. Since carbohydrate storage is relatively small, there is a greater tendency to mobilize protein to supply similar molecular fragments.

DISCUSSION

Two factors appear to be involved in the longer survival of the fat-fed rats in the present experiments: (1) a decreased destruction of structural tissue and (2) a reduced amount of voluntary activity.

The decreased destruction of structural tissue during any given period of fasting was due in part to the reduced voluntary activity. This is probably not the entire explanation, however. The early course of the blood sugar level would indicate that in all probability during the first part of the fast a different metabolic mixture was burned by the animals previously on the different diets. In the case of the carbohydrate group, this involved greater carbohydrate and protein and less fat breakdown. The greater utilization of fat and the consequent sparing of body protein during forced exercise probably accounts for the greater work which the fat-fed rats were able to do before exhaustion. This beneficial effect of a fat diet has also been observed by Deuel et al. ('47) in connection with a high fat diet fed ad libitum.

Differences in glucose utilization, measured by glucose tolerance tests or glycosuria after ingestion of carbohydrate, have been observed by many workers since Hofmeister wrote of "hunger diabetes" as far back as 1890. The literature has been reviewed by Chambers ('38) and by Peters ('45). The effects of a high fat diet and of fasting were formerly thought to be the same, however. The present work demonstrates that the effects of a high fat or high carbohydrate diet persist over a large part of the subsequent fasting survival

period, and are therefore superimposed upon the metabolic changes due to fasting alone.

The recent report of Lundbaek and Stevenson ('48) indicates that the voluntary muscle of a carbohydrate-fed rat utilizes glucose *in vitro* at twice the rate of that of the fat-fed animal when both are exposed to the same environment. Apparently this excess utilization is for energy, since glycogen storage was the same in both groups. Gilmore and Samuels ('48) of this laboratory have confirmed the difference in glucose utilization by diaphragmatic muscle and have shown that a similar difference prevails in the rate of glycogen disappearance when diaphragms from carbohydrate- or fat-fed rats are incubated aerobically in a glucose-free medium. It would appear, therefore, that the local cellular metabolic system is affected.

The increase in the blood sugar level of the previously fat-fed rats during exercise is probably explained by the stimulation of glucose formation from glycogen due to epinephrine release, and by the stimulating effect of exercise on the release of the 11-oxygenated steroids from the adrenal. These increase the breakdown of protein to carbohydrate and at the same time decrease glucose oxidation. Probably both types of hormones were released in both groups of rats, but in the fat-fed group the oxidation of carbohydrate was already decreased at the expense of fat; the glucose mobilized by hormones therefore accumulated. In the carbohydrate-fed group the oxidation of glucose was sufficient to leave the animal in glucose balance.

The marked effect on voluntary activity which followed the change from a mixed diet to one having one major source of energy raises the question of the influence of mixed diets on exercise and survival during fasting. Perhaps the omnivore is in an optimum state when the diet is a mixed one rather than when any single foodstuff predominates. If, however, one foodstuff is to constitute the major source of energy, it would appear that chances for survival under fasting and stress are best when a high fat diet has been eaten.

CONCLUSIONS

With the rat as the experimental animal the administration of a high fat diet (fat furnishing 80% of calories) led to longer survival during subsequent fasting and the ability to do greater amounts of work before exhaustion than a high carbohydrate or high protein diet fed in equicaloric amounts. The protein-fed rats survived for the shortest period.

The difference between the fat and carbohydrate groups could be accounted for on the basis of the difference in the metabolic mixture used and the lower voluntary activity of the fat-fed animals. The early deaths of rats previously on a high protein diet were not explained by these experiments.

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MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1949 Award of \$1000, established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the spring of 1949.

The Award will be given to the laboratory (non-clinical) or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year, January 1 to December 31, the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1948 must be in the hands of the Chairman of the Nominating Committee by January 15, 1949. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

HAROLD H. WILLIAMS
Cornell University, Ithaca, N. Y.

CHAIRMAN, NOMINATING COMMITTEE

BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1949 Award of \$1000, and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the judges may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1949. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 15, 1949. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

JAMES M. ORTEN
*College of Medicine,
Wayne University,
Detroit, Michigan*

CHAIRMAN, NOMINATING COMMITTEE

OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of \$1000, established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1949 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 15, 1949.

D. W. WOOLLEY
*Rockefeller Institute for
Medical Research, New York, N. Y.*

CHAIRMAN, NOMINATING COMMITTEE

MAGNESIUM IN THE NUTRITION OF THE RABBIT¹

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FIVE FIGURES

(Received for publication July 22, 1948.)

The symptoms developed during magnesium deprivation in the rat have been well described (Kruse, Orent and McCollum, '32; Tufts and Greenberg, '38). Because of the anatomical and physiological character of herbivorous animals, their dietary requirements are somewhat different from those of the rat (Olcese, Pearson and Schweigert, '48).

The development of a purified diet supporting nearly normal growth in the rabbit, which has a herbivorous dietary habit (Wooley and Sebrell, '45), and the small size of this animal have made possible the further study under laboratory conditions of the dietary requirements of herbivora. The studies reported here were conducted in order to ascertain the effect of magnesium deficiency on rabbits and to obtain information on the quantitative requirement of these animals for magnesium.

EXPERIMENTAL

Weanling New Zealand white rabbits of both sexes, 5 to 6 weeks of age and weighing from 0.6 to 1.3 kg, were used in these experiments. Food and distilled water were given ad

¹ This work was supported in part by a grant from the Dow Chemical Company, Freeport, Texas, through the Texas A. and M. Research Foundation. Acknowledgments are made to Patricia Sparks for technical assistance with some of the work.

libitum except when the rabbits became diarrheal, at which times the water supply was limited to about half that normally consumed. The rabbits were weighed weekly during the experimental period and were confined to wire-bottom cages in groups of three or 4.

Blood samples were obtained from the rabbits by cardiac puncture without anesthesia. The plasma and whole blood magnesium were determined by the method described by Kunkel, Pearson and Schweigert ('47). Serum calcium was determined by the method of Kramer and Tisdall ('21) as modified by Clark and Collip ('25). Hematocrit readings were made with the use of Wintrobe hematocrit tubes centrifuging at about 3500 r.p.m. for one hour.

As a result of the simultaneous investigation of the adequacy of various purified diets for the rabbit and of the necessity of preparing diets as low in magnesium as practicable, three diets were used. The composition of these diets is shown in table 1. Since facilities for refrigeration were limited, the rations were generally mixed in lots of 5 kg or less in order to avoid rancidity. In the later series of these experiments, where graded levels of magnesium were required, the diets containing the intermediate levels were prepared by mixing proportionate amounts of the rations containing the extreme levels. More than 70 rabbits were used in these investigations.

RESULTS AND DISCUSSION

The first experiment was an exploratory one to determine the adequacy of a diet containing a 1:20 liver concentrate as the source of unknown vitamin factors needing further investigation in studying the requirement of the rabbit for magnesium. A group of 6 rabbits was fed diet 10, which contained approximately 6 mg of magnesium per 100 gm (determined by the method of Lindner, '44). A similar group of 6 animals served as a control and received this diet supplemented with 40 mg of magnesium per 100 gm of diet.

After 7 weeks the performances of both groups were not significantly different. One animal in the group receiving the low magnesium diet died during the 4th week, after severe diarrhea, but later results indicated that this could not be specifically attributed to magnesium deprivation. The mean rate of gain for the group fed the low level of magnesium was 165 gm per week with a standard deviation of ± 16 gm, as

TABLE 1
*Composition of experimental diets*¹

INGREDIENTS	DIET NUMBER		
	10	13	17
	<i>gm</i>	<i>gm</i>	<i>gm</i>
Casein	20	22	22
Peanut oil	10	8	.
Corn oil (Mazola)	.	.	8
Cerelose	47.9	53.9	51.9
Cellulose	12	12	12
Salts IV-A ²	3.6	3.6	3.6
Wood flour	2	.	.
Liver extract, 1:20 (Wilson and Co.)	4	.	2
Fortified cod liver oil	0.5	0.5	0.5

¹ The diets were supplemented with the following vitamins in mg/kg: choline chloride 2000, niacin 200, inositol 100, mixed tocopherols 150, 2-methyl,1,4-naphthoquinone 0.75, pyridoxine 7, thiamine 7, riboflavin 7, and calcium pantothenate 15, with the exception that the two last vitamins were not added to diet no. 10.

² The "salts IV-A" mixture was prepared according to the formula for salts IV described by Hegsted et al. ('41), except that the magnesium sulfate was omitted.

compared with 194 gm per week with a standard deviation of ± 61 gm for the control group. Because of the wide variability in the rates of gain of the animals in the control group, the difference here was not considered significant. None of the symptoms characteristic of a magnesium deficiency in other species was observed.

At this stage of the experiment it appeared that the magnesium level of the basal diet was adequate to meet the needs of the rabbit. The males therefore were sacrificed and the

magnesium content of the plasma and whole blood cells determined. A significant difference in these values for the two groups was found. The mean magnesium level of plasma for the males receiving 6 mg magnesium per 100 gm of diet was 1.0 mg per 100 ml, as compared with 1.3 mg per 100 ml for the controls. The mean magnesium contents of the erythrocytes were 6.9 mg and 9.7 mg per 100 ml for the respective dietary treatments.

The 5 females, three in the group receiving the low magnesium diet and two in the control group, were continued on the dietary regimens until all the animals in the deficient group died. By the 10th week a definite decrease in the growth rate of the deficient animals was noted. They began to show alarm and apprehension at the sound of unusual noises. During the 16th week one rabbit suffered a convulsive seizure in which it ran frantically about the cage, and fell on its side in tonic-clonic spasms with eyes dilated and bulging and vasoconstriction of the cutaneous system. When the convulsive seizure ended the animal lay exhausted on its side, but complete recovery occurred in about half an hour (figs. 1-4). Similar seizures were observed in the other deficient animals and the same pattern was noted. One of these rabbits survived 18 observed audiogenic convulsions.

The deficient females survived from 20 to 25 weeks on diet 10, containing 6 mg of magnesium per 100 gm. A loss of weight and a generally unthrifty appearance became evident during the latter part of the experimental period. After the 18th week attempts were made to breed the animals to normal males. Several tries were required in each case before the male was accepted. Death in all three deficient animals followed 8 to 10 days after mating. Post-mortem examination of one animal revealed that ovulation had taken place and was followed by apparent fertilization and fetal implantation, death and resorption.

The two control females continued to grow normally, as evidenced by the fact that from the 8th to the 14th week the average growth was 155 gm per week as compared with simi-

lar females which gained 111 gm per week while fed a commercial stock diet. From this it appeared that 40 mg of magnesium per 100 gm of diet fed under the conditions of this study were adequate to meet the requirements of the growing rabbit.

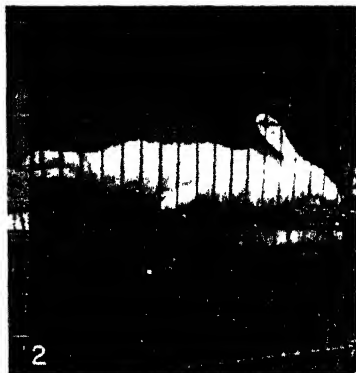


Fig. 1 Magnesium-deficient rabbit apparently normal in appearance before convulsions.

Fig. 2 The same rabbit immediately after the onset of convulsions induced by the sound of an air blast.

Fig. 3 The rabbit immediately following the cessation of convulsions.

Fig. 4 About 5 minutes after the convulsions, the rabbit showing a progressive recovery.

Since the magnesium content of diet 10 appeared to be too high to produce an acute deficiency, a second experiment was designed using diet 13, which contained less than 1 mg of magnesium per 100 gm. Three groups of 5 rabbits each were fed diets containing 40, 6, and less than 1 mg of magnesium in 100 gm of the respective diets. Two of the rabbits in each of the low magnesium groups and one in the group receiving

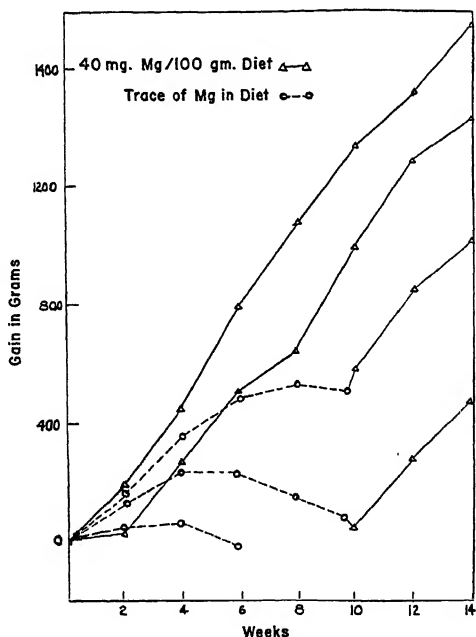


Fig. 5 Effect of magnesium deficiency and the subsequent feeding of magnesium on growth of rabbits.

40 mg of magnesium in 100 gm of diet died after severe diarrhea during the third and 4th weeks.

The symptoms resulting from magnesium deprivation in this experiment seemed to be somewhat more severe than in the preceding one. Retardation of growth was evident by the third week. Convulsions could be induced by the sound of escaping air in the 5th week. A loss of weight and extreme emaciation soon followed the onset of convulsions. By the

9th week all but two animals in the deficient groups were dead. Gross examination of the kidneys of the dead animals revealed a mottled, bloody appearance. Whole blood magnesium of the deficient rabbits after 5 weeks on the experimental diet showed a very significant decrease to a mean value of 3.4 mg magnesium per 100 ml, as compared with 5.5 mg per 100 ml of the blood of animals receiving 40 mg of magnesium per 100 gm of diet. Values as low as 1.8 and 2.4 mg magnesium per 100 ml of whole blood of deficient animals were recorded after the 6th week of the experimental period. Vasodilatation and hyperemia, the symptoms reported to be peculiar to magnesium deficiency in the rat and dog, were not observed in any of the rabbits fed diets low in magnesium.

During the 10th week the two surviving animals on the deficient diets were changed to the diet containing 40 mg of magnesium per 100 gm. There was an immediate response in the rate of growth (fig. 5).

Quantitative requirements

The results of these experiments indicate that the quantitative requirement of the rabbit for magnesium under the conditions described is somewhere between 6 and 40 mg per 100 gm of diet. Studies were conducted in which graded levels of magnesium as the sulfate form were fed, to determine the approximate requirement of the rabbit for magnesium and to obtain information on the subacute deficiency. Other investigations on the adequacy of various purified diets (Kunkel, Simpson, Pearson, Olcese and Schweigert, '48) indicate that factors in the liver concentrate are required for maximum growth and general well-being of the growing rabbit. Liver concentrate was therefore included in diet 17, which was the diet used during the subsequent phases of this study.

The criteria used for the adequacy of the magnesium intake were clinical manifestations, growth and the magnesium content of the whole blood.

Ten levels of magnesium, ranging from 7.5 to 47.5 mg per 100 gm of diet were fed. The experimental period was 10

weeks. Blood was drawn at the second, 4th, 6th, 8th and 10th week for determination of the magnesium content. There was a progressive decline to the 8th week in the magnesium content of the blood of rabbits receiving 25.0 mg or less of magnesium per 100 gm of diet: the decline was more severe in the rabbits on the lower levels of magnesium intake. There was no significant difference between the blood magnesium values at the 8th and 10th weeks.

Rabbits fed a diet providing 17.5 mg of magnesium per 100 gm of diet were subject to audiogenic convulsions and other manifestations characteristic of a magnesium deficiency. The growth rate of rabbits receiving diets containing 17.5 mg of

TABLE 2

Comparison of the effect of magnesium deprivation on the magnesium content of whole blood and plasma of rabbits

EXPERI- MENTAL PERIOD	ON DIETS WITH MAGNESIUM CONTENT			
	Less than 20 mg/100 gm		37.5 to 47.5 mg/100 gm	
	Whole blood	Plasma	Whole blood	Plasma
<i>(all values are mg magnesium/100 ml blood or plasma)</i>				
10 weeks	3.15 \pm 0.75 (25) ¹	1.00 \pm 0.22 (10)	4.36 \pm 0.92 (16)	1.61 \pm 0.38 (8)

¹ Mean value and standard deviation. Number in parentheses indicates number of animals.

magnesium per 100 gm was 119 gm per week, as compared to gains ranging from 145 to 165 gm with 22.5 mg or more of magnesium.

The average magnesium content of the blood of rabbits fed diets containing 27.5 mg or less of magnesium per 100 gm was less than 4.0 mg per 100 ml, whereas when the intake was 32.5 mg of magnesium per 100 gm of diet the level in the blood was 4.6 mg per 100 ml or higher.

Data on the magnesium content of the whole blood and plasma after 10 weeks on the experimental diets are summarized in table 2. Those rabbits fed 20 mg or less of magnesium per 100 gm of diet have been averaged together and those fed 37.5 mg or more similarly averaged. It is recognized

that rabbits fed levels of magnesium between these levels may have been near the borderline of adequacy of magnesium intake. A diet providing 20 mg or less of magnesium per 100 gm of ration results in levels of magnesium in both the plasma and whole blood that are significantly lower than those on diets providing 37.5 mg or more of magnesium per 100 gm.

The level of calcium in the blood was not affected by the magnesium deprivation. The mean value of serum calcium was 15.5 mg per 100 ml, with no difference among the various groups. Slightly lowered blood cell volume was noted in the more deficient animals.

Since there were no clinical or biochemical manifestations of a magnesium deficiency on diets providing 32.5 mg or more magnesium per 100 gm, the data indicate that the magnesium requirement of the rabbit is between 30 and 40 mg per 100 gm of diet when the magnesium is furnished as the sulfate salt. This level is somewhat higher than that of 20 mg of magnesium per 100 gm of diet which appears to satisfy the requirements of the rat (Kunkel and Pearson, '48).

SUMMARY

Weanling rabbits fed a diet deficient in magnesium exhibit within a period of three to 6 weeks a syndrome involving hyperexcitability, convulsions, hypomagnesemia, and retardation of growth. The addition of magnesium to the diet of rabbits that have ceased to grow results in a prompt resumption of growth. The vasodilatation characteristic of magnesium deficiency in rats and dogs was not observed in rabbits.

The magnesium content of the blood of rabbits fed a diet adequate to prevent symptoms of a magnesium deficiency was approximately 4.4 mg per 100 ml, while the level for the plasma was about 1.6 mg per 100 ml. On a diet containing 20 mg or less of magnesium per 100 gm of diet the level in the blood shows a progressive decline to about the 8th week of about 3.1 mg per 100 ml for whole blood and 1.0 mg per 100 ml for plasma.

The magnesium requirement of the rabbit appears to be between 30 and 40 mg per 100 gm of diet.

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VOLUNTARY CALORIC INTAKE OF THE GROWING RAT

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TWO FIGURES

(Received for publication July 6, 1948)

It is a common observation in laboratories engaged in rat growth studies that animals growing poorly ingest less food. In repletion studies such as those described by Cannon and co-workers ('44) animals showing poor weight recoveries likewise eat smaller quantities of food.

The fact that animals growing poorly do ingest less food has prompted numerous modifications in technics for the study of ration quality wherein attempts are made by one means or another to equalize food intakes. A strong positive correlation exists between weight gain and food intake. For example, Harte and Travers ('46) have analyzed data for 279 male rats fed diets of constant composition except for protein quality and found a correlation coefficient of $+0.82$ between these variables, while similar data on 61 rats fed a diet containing casein showed a correlation coefficient of $+0.67$. Hegsted and Worcester ('47) studied similar data after logarithmic transformation of the variables and reported a correlation coefficient of $+0.83$ between the transformed variables.

Examination of data in the literature and preliminary observations suggested the existence of a constant relationship between the voluntary caloric intake of growing rats fed ad

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libitum and the size they attained. The experiments to be described in this report were carried out in an attempt to establish this relationship for the growing rat.

In part of the experimental work the design was such that analyses of variance enabled the drawing of collateral conclusions on the roles of sex and litter as well. It is worthwhile to emphasize, as Addis and associates ('48) have done, how valuable the application of modern small-sample statistical theory to experimental data can be. By means of these techniques, various aspects of the data of a single well-designed experiment may be evaluated and hypotheses tested without setting up a series of individual comparisons. In this way the efficiency of experimentation is markedly enhanced and the same amount of laboratory work may be made to yield substantially more information.

EXPERIMENTAL

Two series of tests were carried out. In the first, 7 litters born over a period of about one month by breeders from our own colony (Sherman strain albino rats, originally obtained from Rockland Farms) were used. The litters totalled 17 males and 21 females. These animals were weaned 21 days after birth and fed semi-synthetic Ration A (table 1) ad libitum. After 9 weeks all animals except those from one litter were transferred to Ration B (table 1), on which they were maintained for an additional three weeks. The ration compositions were designed to make the ration of protein calories to total calories the same.

In the second series of experiments weanling males, purchased to a weight specification, were used. The general technic and ration composition have been described by Harte et al. ('47), except that biotin was included in all rations at a level of 0.1 mg per kilogram and a series of different proteins² were substituted for the casein used in the experiments

² Most of the proteins used in this part of the study were provided by the Bureau of Biological Research of Rutgers University as part of its program for the collaborative study of protein assay methods. The results of growth tests with these proteins will be reported elsewhere.

cited. Appropriate adjustments in the cornstarch content of these rations made them isonitrogenous and they were assumed to be isocaloric.

Caloric intakes were calculated from food intakes and the caloric values of the rations; the latter were estimated by assuming that protein and carbohydrate yield 4 Cal. per gram and fat, 9 Cal. per gram. While calorimetric determinations might have been preferred, these assumptions were considered sufficiently accurate for the present purposes, particularly in the light of the uncertainties involved in the estimation of surface areas.

TABLE 1
*Ration composition*¹

COMPONENT	RATION		COMPONENT	RATION	
	A	B		A	B
	%	%		%	%
Dried skim milk	8.38	7.25	Cornstarch	50.3	57.0
Casein	13.4	11.6	Lard	9.0	1.0
Lactalbumin	3.35	2.9	Ruffex	...	5.0
Malt sugar	8.37	7.25			

¹ In addition, each kg of ration contained salts, 40 gm; wheat germ oil, 10 gm; cod liver oil, 10 gm; liver extract concentrate (Wilson's Liver Extract Concentrate, 1:20, was used) 10 gm; choline chloride, 1.8 gm; thiamine hydrochloride, 5 mg; riboflavin, 8 mg; pyridoxine, 6 mg; calcium pantothenate, 13 mg; and niacin, 13 mg.

Computed Calories per gram: 4.47 for ration A, 3.84 for ration B.

The salt mixture used had the same composition as that described by Phillips and Hart ('35) but was modified by the addition to their formula of 0.2 gm of potassium alum and 1 gm of sodium fluoride.

In the handling of the data accumulated during the experimental periods, surface areas of the individual animals were estimated from the formula $A = 11.36 W^{2/3}$, reported by Carman and Mitchell ('26). Average areas for each animal during any given week were obtained by averaging the areas corresponding to the weights at the beginning of the week and at the end of the week.

RESULTS

The average growth of the animals in the 7 litters used in the first experiment, as evidenced by changed body weight, is shown in figure 1. This growth was quite satisfactory, averaging 4.8 gm per day for the males and 2.8 gm per day for the females during the 63 days on Ration A. These data fit straight lines in the log weight-reciprocal age coordinate system proposed by Zucker and Zucker ('42), with slopes differing only slightly from those described as optimum. For

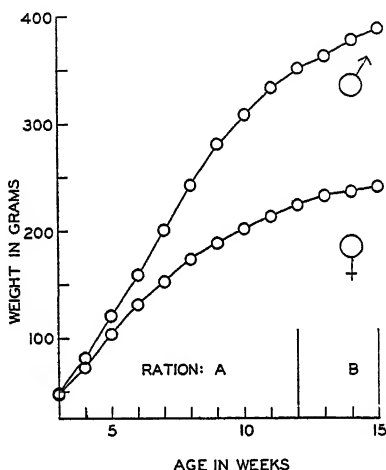


Fig. 1 Growth curves for male and female rats fed ration A through the 12th week of life and ration B thereafter. Of the 17 males and 21 females fed ration A, 15 males and 17 females were continued on ration B.

females the slope is 2.74 compared to Zucker's 2.83; for males, 3.54 compared to Zucker's 3.71. Evidence of the excellence of fit to linearity is found in the values of the rectilinear correlation coefficients between reciprocal age and log weight, which are -0.9984 for females and -0.9958 for males.

While analyses of variance indicated very clearly that the 9-week gains for both males and females had substantially smaller variance within litters than between litters, this significant difference, which would favor the use of litter mates, disappeared when differences in initial weight were taken

into consideration by application of the methods of covariance. Differences in initial weight, sex being the same, had a greater influence on weight gain than whether or not the animals were litter mates.

In figure 2 are plotted, for males and females separately, the voluntary calorie intakes of these animals calculated as Calories per square decimeter per day. The data consistently indicate a higher value for the 5th week of life than for the 4th, but after the 5th week a steady decrease was observed, the intake approaching as an approximate limit a value of between 12.5 and 13 Cal. per square decimeter per day for the animals of both sexes.

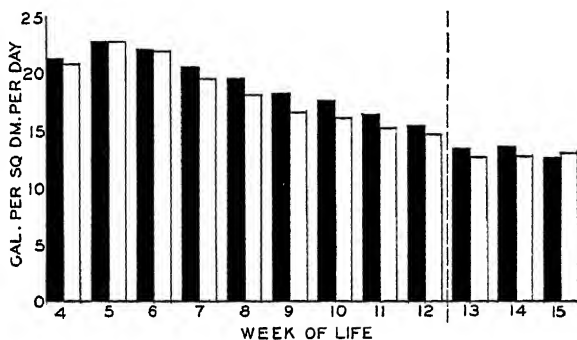


Fig. 2 Bar chart giving mean voluntary caloric intake in Calories per square decimeter of body surface per day for weekly intervals. Solid bars represent males; open bars, females. Values to the left of the dashed line were obtained with ration A; to the right, with ration B. Ration A was fed to 17 males and 21 females; ration B to 15 males and 17 females.

A complete analysis of variance of these data, summarized in table 2, showed that the observed differences from week to week during both the first 9 weeks on the ration containing 4.47 Cal. per gram, and the last three weeks on the ration containing 3.84 Cal. per gram, were statistically highly significant. On the other hand, during the first 9 weeks the difference in caloric intake between the sexes was highly significant while during the last three weeks it was not significant. Similarly, for both males and females during the first 9 weeks

differences between litters were significant as compared to differences within litters, while during the last three weeks the inter-litter differences were not significant for either sex.

The second series of experiments were carried out with rations practically identical with each other except for protein source. At the end of the 5-week feeding period the animal weights reflected the differences in protein quality by

TABLE 2
Ad libitum caloric consumption—analysis of variance

SOURCE OF VARIATION	RATION WITH 4.47 CAL. /GM FED 1 TO 9 WEEKS INCLUSIVE		RATION WITH 3.84 CAL. /GM FED 10 TO 12 WEEKS INCLUSIVE	
	Degrees of freedom	Mean square	Degrees of freedom	Mean square
Between weeks	8	296.349 ¹	2	8.061 ²
Within weeks	333	2.412	93	1.385
Individuals	37	9.757	31	2.073
Sexes	1	79.190 ²	1	3.508
Within sexes	36	7.828	30	2.025
Males	16	6.959	14	1.231
Inter-litter	6	13.069 ³	5	0.884
Intra-litter	10	3.294	9	1.424
Females	20	8.524	16	2.720
Inter-litter	6	20.826 ²	5	4.827
Intra-litter	14	3.252	11	1.763
Discrepancy	296	1.494	62	1.040

¹ F value exceeds that tabulated for $p = 0.001$.

² F value exceeds that tabulated for $p = 0.01$.

³ F value exceeds that tabulated for $p = 0.05$.

ranging from 62 to 169 gm. Voluntary caloric intake in Calories per square decimeter per day were computed from the data as before. The analysis of variance of these data indicated that the possibility that the differences in unit caloric intake observed with time might be due to chance is extremely small (p less than 0.001). Far more striking is the observation that the differences in caloric intake with different rations

are not statistically significant (p greater than 0.20). In table 3 the averages for the unit caloric intakes of the animals (all males) receiving these different rations are given, as well as averages of the observed weight ranges and gains.

TABLE 3

Weight gains and average caloric intakes of growing rats on rations containing proteins of different qualities ($N \times 6.25 = 10\%$)

PROTEIN SOURCE	BODY WEIGHT		CALORIC INTAKE DURING WEEK					NO. OF RATS
	Initial	Gain ¹	1	2	3	4	5	
	<i>gm</i>	<i>gm</i>	<i>Cal./day/dm² of body surface</i>					
Casein I	56.6 \pm 1.8 ²	77.6 \pm 3.9	21.2	18.4	18.3	17.5	16.5	10
Casein II	56.7 \pm 1.5	71.3 \pm 2.6	20.4	17.6	16.6	16.9	17.7	9
Beef	54.0 \pm 1.6	85.7 \pm 6.3	20.2	19.2	18.7	18.1	17.1	9
Whole egg	52.6 \pm 2.1	105.4 \pm 4.1	20.9	19.9	17.9	17.1	16.8	9
Peanut flour	55.3 \pm 1.7	81.3 \pm 3.0	21.0	19.4	17.3	17.9 ³	17.9 ³	10
Wheat gluten	56.9 \pm 1.8	14.4 \pm 1.8	19.3	18.4	17.7	17.8	16.5	9
Weighted mean			20.5	18.8	17.8	17.6	17.1	

¹ Weight gains are those recorded after ad libitum feeding for 5 weeks.

² Indicated limits represent standard errors of the reported means.

³ During the latter part of the 4th week and the beginning of the 5th week these animals received protein at a somewhat lower level due to an error in making up one batch of this ration.

When these data were calculated on the basis of Calories per 100 gram of body weight per day, the analysis of variance showed the differences between rations to be very highly significant. This recalls the importance of the evidence linking metabolic phenomena with body surface, as reviewed by Brody ('45). Data were also available, but are not reported here, on a similar ration containing egg white. It appeared that animals fed this ration consumed significantly fewer Calories per square decimeter per day, although they grew very well. Additional study will be required to verify this observation.

DISCUSSION

The voluntary eating habits of the growing rat as elucidated by these experiments show surprising uniformity from ani-

mal to animal. That young males will voluntarily ingest food equivalent to more energy per unit of surface area than females of the same age might be expected. In humans, boys tend to have a higher basal metabolism than girls and this difference between the sexes persists well into maturity but seems to disappear after about 60 years of age. The analogous situation with respect to rats has been studied by Mitchell and Carman ('26).

The occurrence of a maximum in voluntary unit caloric intake during the 5th week of life in the growing rat finds its parallel in the presence of a maximum in the relation between resting metabolism and age (Brody, '45). This latter maximum seems to occur somewhat later, coinciding roughly with an age of 40 days. In some measure, then, the changing voluntary caloric intake reflects, or is reflected by, a changing unit resting metabolic rate.

The energy requirements of the mature rat have been estimated by various workers. Benditt et al. ('48) have shown that the utilization of a constant quantity of protein is restricted when the caloric intake falls below about 12.4 Cal. per square decimeter per day in the protein-depleted rat. Bosshardt et al. ('46) found that the critical caloric value for the growing rat is approximately 12.5 Cal. per square decimeter per day, averaged over a 42-day period. In the light of the changing unit demand and the nature of the relation between weight and area, this period may be too long to yield an entirely valid average. During the most active period of growth after weaning our rats voluntarily ingested food equivalent to almost twice that number of Calories, but their unit consumption tended to decrease quite regularly and apparently to stabilize in the vicinity of 12.5 to 13 Cal. during the 14th and 15th weeks of life.

No correction was made to accord with the viewpoint of Cherkin ('48) that dietary nitrogen utilized exclusively for protein functions should not be included in estimating the energy content of the diet. Since some of the dietary fat and carbohydrate may be retained as glycogen or tissue fat, cor-

rection in the estimation of their contributions to the total Calories would also be required. Whether dietary calories from any source are released as heat promptly after ingestion or are stored to become potential Calories does not affect the over-all bookkeeping necessary to establish the status of caloric balance. Stored foodstuffs take the form of potential Calories, rather than realized Calories, and these considerations make Cherkin's argument misleading except in a highly specialized, short-term sense.

Of course if appetite is a factor there is no reason to suppose that any relation need exist between the animal's caloric requirements and the number of calories which it will voluntarily ingest. There is some indication that this may represent the state of affairs when diet composition is markedly varied. Preliminary observations on adult rats receiving protein-free rations indicate that these animals will voluntarily ingest fewer calories than similar animals offered rations containing 5 or 10% casein. Also, when the composition of the diet is changed — e.g., from 4% fat to 20% fat — the voluntary caloric intake rises enormously and persists at a high level for as long as two weeks before dropping to the previously attained plateau value. The situation does not seem to be simple because to some extent, at least, the animal eats to satiety. For a mature animal maintained on a given diet satiation might require, let us say, 18 gm of ration daily. When such a diet is replaced by one of higher caloric content, approximately the same bulk will be required initially to provide the same degree of satiety and as a result the caloric intake will rise until metabolic adjustments take place.

Particular interest attaches to the apparent uniformity of voluntary caloric intake on rations differing only in their protein quality. Under these conditions, it would appear that the primary purpose in eating is to satisfy energy requirements and that their satisfaction is a function of the age and sex (which determine the unit requirement) and the size of the animal. Animals of the same age and sex starting at the same size will initially ingest equivalent calories, both in

totality and per unit of surface area. If as an accidental corollary to the ingestion of these calories some of the animals become larger, they will voluntarily ingest more calories, simply because there are a larger number of units of surface area seeking caloric satisfaction. It would appear, then, that weight gain is merely incidental to the satisfaction of energy demands. So long as the animals are receiving rations of essentially constant composition, they will ingest just sufficient food to satisfy their caloric requirements per unit of surface area. Differences in food consumption observed with different rations will, under these conditions, merely reflect differences in animal size.

In carefully conducted studies with adult dogs, Cowgill ('28) has shown that these animals voluntarily regulate their food intakes to satisfy their energy requirements and, other things being equal, do so in obedience to the so-called surface area law. The present findings with the growing rat are in full accord with Cowgill's speculative extrapolation of his findings to the feeding of infants.

These considerations offer an explanation for the observation previously noted that if the protein quality is poor the animals ingest less food. An unsuitable assortment of amino acids, reflecting deficiencies in the protein's pattern or lack of digestibility, does not permit the formation of new tissue. Failure of size to increase has as its consequence the reduction of total caloric intake.

With respect to rats and proteins, one may well say "If it's a poor protein, they won't eat well," but it cannot be emphasized too strongly that no evidence has been presented for the reciprocal statement, "If they won't eat well, it's a poor protein." Strong temptation to invert the first statement into the second has become the basis of reckless inferences for which no justification exists.

On cursory examination it would appear that the 9-week weight gains are more uniform for animals of the same sex and litter than for animals of the same sex chosen randomly with respect to litter. This conclusion, however, does not take

into consideration variations in initial weights in litter mates of the same sex. When appropriate adjustments are made for the divergence in weaning weights of litter mates of the same sex, then there is just as much uniformity among animals of the same sex, age and weaning weight without regard to litters as is found in animals of the same sex and litter. Our data definitely suggest the possibility that the former criteria of selection might provide even more uniform animals. This is in agreement with the implicit hypothesis of Zucker's growth equation (Zucker and Zucker, '42), which suggests that the growth curve of an optimally-fed animal is uniquely determined by its weaning weight. For any given strain the slope term of Zucker's equation is given as a constant not subject to inter-litter variation.

SUMMARY

1. The voluntary food consumptions of 21 female and 17 male rats from 7 litters were studied during the first 12 weeks after weaning.

2. During the second week after weaning the voluntary caloric intake per unit of surface area increases over that observed in the first week; and thereafter it decreases, leveling off at a value between 12.5 and 13 Cal. per square decimeter per day.

3. To some extent this equilibrium value may be related to the proximate composition of the ration.

4. Given rations of the same proximate composition but differing in their ability to promote growth as a consequence of differences in protein quality, the voluntary caloric intake per unit of surface area per day was constant during 5-week growth studies, regardless of the quality of the protein component, when the protein source was casein, beef, whole egg, peanut flour or wheat gluten.

5. This apparent constancy of unit caloric intake emphasizes that the primary urge in the ingestion of food is the satisfaction of energy requirements and that, other things

being equal, if growth ensues it is, within limits, an accident of the make-up of the ration.

6. The data indicate that animals will be more uniform in their growth responses if they are selected on the basis of sex and weight at a given age than if the latter factor is ignored in favor of the criterion that they be litter mates.

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THE EFFECT OF THE COMPLEX-FORMING AMORPHOUS SODIUM PHOSPHATES ON THE CALCIUM RETENTION OF RATS ¹

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ONE FIGURE

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INTRODUCTION

The demonstration that tartrates (Hamilton and Schwartz, '33) and citrates (Hamilton and Dewar, '37; Shohl, '37) prevent rickets when added to a rickets-producing diet has resulted in several investigations designed to explain this action. Originally it was suggested that acid-base factors were largely responsible (Hamilton and Schwartz, '33). This was confirmed in part by the subsequent work of Shohl ('37), who nevertheless showed that this hypothesis was inadequate, as did Hamilton and Dewar ('37). The former stated that although "the acid reaction and alkali ash factors are necessary," an investigation of the complex-forming properties of the tartrates and citrates would be of value. The latter investigators suggested that the citrates and tartrates may lead to a decrease in the availability of calcium, either by complex formation or by the formation of insoluble calcium salts other than phosphate, thereby increasing the probability of the absorption of phosphates and in this manner preventing rickets. They showed too that the salts of citric and tartaric acids are

¹ Taken from a thesis submitted by M. K. Borenstein in partial fulfillment of the requirements for the degree of Master of Arts, University of South Dakota, 1947.

more effective in rickets prevention than the free acids. The work of Hathaway and Meyer ('39) indicates that the potassium salts are more effective than the sodium salts. Day ('40) suggests that the "beneficial effect of citrates is mediated through the formation of a calcium complex which interferes less, if at all, with the utilization of phosphorus in phytin."

In view of the strong probability that complex formation may play an important part in the effects of the citrates and tartrates, it seemed of interest to determine the effects on calcium metabolism of other calcium complex-forming materials. Of the complex formers now known, amorphous sodium phosphate is probably the most effective. Consequently, the calcium and phosphorus retentions of rats on the Steenbock-Black ('25) diet to which amorphous sodium phosphate was added were determined, as well as similar retentions of rats on the same basal diet to which was added a crystalline, soluble, non-complex-forming phosphate of approximately the same composition.

EXPERIMENTAL METHODS

Rats and metabolism cages

Male rats (Sprague-Dawley, Inc., Madison, Wis.) between the ages of 24 and 28 days were used. The metabolism cages, cylindrical and made of wire mesh, were similar in principle to those described by Brooke and Smith ('33).

Collection of samples

The collection of urine and feces was carried out essentially as described by Brooke and Smith ('33), with the following modifications: (1) The acetic acid washed filter papers used for collecting the urine were pressed dry on a letter press rather than sucked dry on a Buchner funnel; (2) for extraction, the filter papers were macerated in a Waring-type blender, using the cage washings as the fluid medium. The slurry of macerated filter paper and cage washings was al-

lowed to settle overnight before filtration and washing by suction. The filtrates were evaporated to a convenient volume, transferred quantitatively to volumetric flasks and made up to volume; aliquots were used for calcium and phosphorus determinations.

The efficiency of the extraction procedure was checked by further extraction of the filter paper residue with 2 N HCl. No calcium was found in the hydrochloric acid extract, and phosphorus to the extent of only 0.1% of the total in the urine sample was recovered.²

Occasionally some spilled dried food remained on a filter paper used for the collection of urine. The amount in general was small and would not influence the final retention figures.

Preparation of samples

For the determination of calcium the urine samples required no ashing. Dry ashing of the urine for the determination of phosphorus proved to be less satisfactory than a wet ashing procedure. The desired aliquot was dried in a pyrex digestion tube in a water bath. The residue was heated with 1 ml of concentrated sulfuric acid until charring occurred, then the cautious, drop by drop addition of 2 to 5 drops of perchloric acid while heating was continued served to complete the digestion. During heating the tubes were covered with a cone-shaped piece of pyrex glass about one inch high, with its apex downward and offset so as to return any condensate along the wall of the tube. The base of the cone, which rested on the lip of the digestion tube, was flared unevenly in order to minimize the effects of "bumping."

The feces were ashed by the method of Berggren ('32). The ashed samples were taken up with 1 ml of distilled water and 1 ml of concentrated hydrochloric acid, heated on the water bath for 30 minutes (water and acid being replaced as they evaporated), transferred quantitatively to volumetric flasks, and aliquots taken for analysis.

² No correction was made for this in computing the final results, since the amount is insignificant.

Analytical methods

Calcium was precipitated as the oxalate from aliquots of both urine extract and feces ash by the method of Van Slyke and Sendroy ('29), washed, dissolved in 1 N sulfuric acid, and titrated with 0.01 N potassium permanganate using the modified Tisdall-Kramer method as described by Clark and Collip ('25). The determinations were checked from time to time by the use of solutions of known calcium content.

Phosphorus was determined on the ashed urine and feces by the method of Fiske and Subbarow ('25) using a Coleman spectrophotometer. Phosphates were run in groups of 20 to 40 individual determinations and a standard curve was made for each group. Check determinations were run periodically, using the volumetric method of Lundell and Hoffman ('24).

All analyses were run in duplicate, and in any instance where duplicate determinations of either calcium or phosphorus failed to agree to within 5 parts per 100, both determinations were repeated.

Diets

The Steenbock-Black ('25) rickets-producing diet was used as the basal diet. Amorphous sodium phosphate with a ratio of Na_2O to P_2O_5 of about 1.1 to 1 was used as received.³ Crystalline soluble sodium phosphate with approximately the same ratio of Na_2O to P_2O_5 was purified before use by recrystallization from hot water. The calcium and phosphorus content of each diet was determined by the methods previously indicated. Samples for analysis were obtained in each case by quartering from 330 gm of the well mixed diet. The calcium and phosphorus contents of the diets used are shown in table 1.

As will be noted, there is some slight variation in diet compositions. This is probably due to the fact that each diet

³ The phosphates used in this work were obtained through the courtesy of Calgon, Inc., Pittsburgh, Pa. The amorphous material is commonly known as sodium "hexametaphosphate." The soluble crystalline substance is called sodium "trimetaphosphate." To save space the former will be called Am.P., the latter Cry.P., in this paper.

was mixed separately and that during the course of this work the supply of some of the dietary components was replenished.

General procedure

Each animal was kept throughout the experiment in an individual cage. Cages were cleaned and food and water renewed three times weekly. Once a week each cage was washed down thoroughly with hot distilled water and 2 N acetic acid. The excreta for each rat were combined for a one-week period. Each experiment lasted for 4 weeks. Rats were handled in groups

TABLE 1
Calcium and phosphorus contents of diets used

DIET		Ca	P	Ca/P
		%	%	
1.	Steenbock-Black (a)	1.30	0.33	3.94
	(Five different (b)	1.28	0.35	3.66
	mixes were used (c)	1.19	0.27	4.41
	in the course of (d)	1.17	0.26	4.50
	this work.) (e)	1.15	0.25	4.60
2.	Steenbock-Black + 7% Am.P. ¹	1.19	2.54	0.47
3.	Steenbock-Black + 7% Cry.P. ²	1.05	2.13	0.49
4.	Steenbock-Black + 3½% Am.P.	1.16	1.34	0.87
5.	Steenbock-Black + 3½% Cry.P.	1.14	1.28	0.89

¹ Amorphous material commonly known as sodium "hexametaphosphate."

² Crystalline substance called sodium "trimetaphosphate."

of 12. Six animals were maintained on the phosphate-containing diet under experiment, three were maintained on the same diet supplemented with 30 I.U. of vitamin D for every 5 gm of food consumed, and three were kept on the unaltered Steenbock-Black diet. A preliminary experiment with 6 rats on the basal Steenbock-Black diet was carried out as a check on procedure before any work with the phosphates was begun.

When the 4-week experimental period had elapsed, all rats were killed and x-rayed to determine the presence or absence of rickets.

RESULTS AND DISCUSSION

A summary of the results of the analyses carried out during the course of this work is given in table 2.

A study of the data obtained based on one of the usual tests for significance (Snedecor, '40) indicates the following:

1. The amorphous sodium phosphate used, when added to the Steenbock-Black diet in the amount of 7%, results in greater calcium retention than that observed when the same amount of crystalline phosphate of approximately the same composition is used, although the Ca to P ratios for the diets are almost equal ($t = 6.1$).

2. When the phosphates are added to the diets in amounts of 3½% of the total weight, differences of little or no significance are observed between the calcium retention of the rats on one diet and that of the rats on the other.

3. The ingestion of vitamin D by the rats on the diets containing either 7% or 3½% of the phosphates did not alter the results significantly from those obtained without the added vitamin.

Previous work with the complex phosphates has not been extensive and in general is not strictly comparable to the work herein reported. Shelling in 1932 found that parathyroidectomized rats fed on a low-calcium, high-phosphorus diet developed tetany, while those maintained on a high-calcium, low-phosphorus diet did not show symptoms of this disorder. In addition, he noted that the rats had a tendency to refuse the tetany producing diet, lose weight, and become cachectic. In this connection it was observed that the high-phosphorus diets would induce the tetany symptoms in the rat when the phosphorus was in the form of the ortho- or pyrophosphate; when it was in the form of the metaphosphate or hypophosphate it was ineffective in producing tetany. On the basis of these observations Shelling assumed that the metaphosphate and the hypophosphate are "either inert biologically or excreted unchanged."

The data presented in the present paper indicate that Am.P. (amorphous sodium hexametaphosphate) and Cry.P. (soluble

crystalline sodium trimetaphosphate) are not inert, or at least that they are absorbed from the diet as evidenced by the large increases in urinary phosphorus when they are ingested; that Am.P. is effective biologically is shown by the increase in the calcium retention of rats to which it is fed. Furthermore, fresh samples of either material show only traces of orthophosphate, whereas fresh urine samples from rats fed diets containing either of these substances showed large amounts of orthophosphate, indicating that they are hydrolyzed in the body. Thus, it is probable that the metaphosphate to which Shelling refers was neither Am.P. nor Cry.P. but rather a commercially available insoluble crystalline material which may, in fact, because of its relatively poor solubility, be inert.

Similarly, Fraser and his co-workers ('46) report that the insoluble sodium metaphosphate is far less available when fed to rats on a phosphorus-deficient diet than the soluble orthophosphate. They also found that the orthophosphate is more effective in stimulating the storing of both phosphorus and calcium, particularly when oxalate or ferric ions are present in the diet. This is not too surprising in view of the relative solubilities of the particular phosphates used. These authors also report an experiment involving retentions of calcium and phosphorus on a low-phosphorus diet in the presence of ferric citrate, where a loss of calcium is noted which is obviously due to a loss of soft tissue. Since the diets they used differ markedly in phosphate content from those herein reported, comparison of their results with those obtained in this work is not possible.

In another report involving the addition of Am.P. to the diet of rats, Schwartz and his co-workers ('40) observed the effect of Am.P. treated milk on a group of rats and found that the daily urinary output of calcium and the net calcium retention were increased while the daily fecal calcium was decreased.

Murray ('40), investigating the pharmacological action of the metaphosphates, found that the introduction of Am.P. into the blood stream would result in symptoms such as might

be expected with the removal of calcium ions from the blood. He also showed that when calcium and the amorphous metaphosphate were mixed prior to injection into the blood stream the above effects were not elicited. No symptoms of tetany were observed in the course of this study. It would appear, therefore, that Am.P. is normally absorbed with sufficient calcium to nullify its tetany-producing properties or that it is hydrolyzed during the course of absorption to orthophosphate and after absorption behaves as does the latter.

From these reports and the data obtained in the present investigation it is obvious that the soluble complex phosphates, at least insofar as Am.P. and Cry.P. are concerned, are absorbed and excreted and are physiologically active, in that they appear to exhibit *in vivo* the same properties that they exhibit *in vitro*.

Am.P., which forms complexes with calcium *in vitro*, does increase calcium retention significantly when added to the Steenbock-Black diet in 7% quantities. Cry.P., which does not form complexes *in vitro*, does not have a corresponding effect when added in the same amount. Since both of these materials are neutral in character, the increased calcium retention when Am.P. is used can at present be explained only in terms of its complex-forming properties. Thus in the digestive tract the presence of a sufficient quantity of Am.P. probably results in the formation of a soluble calcium-metaphosphate complex ion,⁴ which is absorbed. Any excess phosphate and calcium are excreted through the kidneys except for the probably insignificant amount excreted through the intestines, the rest being retained.

In the presence of Cry.P., which does not react with calcium, calcium and Cry.P. are absorbed independently. After or during absorption the Cry.P. is hydrolyzed to orthophosphate, the excess excreted, and the remainder retained with calcium.

In the presence of water both substances are hydrolyzed finally to the orthophosphate with a velocity determined by

⁴ The chemistry of these materials is discussed in greater detail by Schwartz et al. ('40).

concentration, pH, temperature and other factors. It is not likely that such hydrolysis takes place to any appreciable extent in the digestive tract since, if it did, no great difference in results between using Am.P. and Cry.P. would have been observed. Furthermore it is not likely, for the reasons previously given, that these substances pass into the blood unchanged. Thus it would appear that they are both absorbed as complex phosphates, with or without calcium as the case may be, and that during or after this process are hydrolyzed to orthophosphate, the excess of which is subsequently excreted.

When only $3\frac{1}{2}\%$ of the phosphates is added to the diets, no significant difference in calcium retention is observed. A possible explanation may lie in the chemical properties of Am.P. It is well known that if Am.P. is carefully added to a calcium solution a gelatinous precipitate forms, possibly $[\text{Ca}(\text{PO}_3)_2]_n$. This is relatively insoluble but is dissolved by an excess of Am.P. When $3\frac{1}{2}\%$ of the latter is added to the diet it is conceivable, therefore, that $[\text{Ca}(\text{PO}_3)_2]_n$ is the predominant result, rather than the soluble complex ion. Since the former is relatively insoluble, no increase in absorption would necessarily result. This would appear to be the case if it is assumed that all calcium not appearing in the feces is absorbed. As might be expected, the addition of vitamin D to the phosphorus-rich diets did not alter the results significantly.

No rickets could be detected by x-ray examination of the rats on any of the phosphate supplemented diets. The change of the calcium-phosphorus ratio by the addition of the phosphates obviously renders the Steenbock-Black diet ineffective in the production of rickets. All control rats on the basal diet without added phosphates did develop rickets.

A severe diarrhea was noted within a few days after the rats were placed on the diets containing both the amorphous and crystalline sodium metaphosphates at a level of 7%. The high concentration of salts, with the probable concomitant dehydration of the gastrointestinal tract resulting in an increase of fluid in the alimentary tract, seems to be the under-

lying cause. Since no diarrhea was observed when the animals were maintained on the diet containing 3½% added phosphates, it can safely be surmised that the phosphate itself does not have a specific effect on the production of diarrhea. In addition to the symptoms of diarrhea, the animals on the diet containing 7% of phosphate excreted urines which had a peculiar reddish tinge. Tests for occult blood proved negative. No analyses for heme compounds were made.

Pathological examination of the visceral organs of the rats in the experiment using 7% amorphous metaphosphate failed to show any abnormalities, either in microscopic sections⁵ of the visceral organs or on macroscopic examination. A cloudy swelling was observed in the tubular epithelium of the kidney, which cannot be considered pathological. Diarrhea in rats when the amorphous sodium metaphosphate amounted to 10% of the diet has been reported (Schwartz et al., '40). The results of pathological examination in that study were essentially the same as those observed in the present investigation. The action of the amorphous metaphosphate seems to be similar in effect to that of any mineral cathartic.

As a check on the experimental methods used, groups of rachitic control rats on the basal Steenbock-Black diet were studied during each experiment. Data were obtained from a total of 18 rats and a summary of the observations made is presented in table 3. The retentions found are of the same order of magnitude as those reported by others, although there are some minor differences. While there was considerable variation in the absolute quantities of calcium and phosphorus retained, the relation between calcium and phosphorus retentions was quite good and all 18 values for the 4-week retentions fell quite close to a curve (fig. 1) whose equation as found by the method of least squares is

$$\text{Log } P = 1.37 \times 10^{-3} \text{ Ca} + 1.67$$

where P = milligrams of phosphorus retained for a 4-week

⁵The authors are indebted to Dr. Ralph L. Ferguson of the Department of Pathology, University of South Dakota School of Medicine, for microscopic examination of the animals.

TABLE 3

*Ca and P retention of 18 rats on the Steenbock-Black diet*¹

WEEK	INTAKE		EXCRETION						RETENTION			
			Ca			P			Ca		P	
	Ca	P	Urine	Feces	Total	Urine	Feces	Total	Absolute	%	Absolute	%
1	530	130	120	325	445	2	96	98	85	16.0	32	24.6
2	551	136	130	346	476	2	97	99	75	13.6	37	27.2
3	565	139	135	368	503	3	106	109	62	11.0	30	21.6
4	543	134	128	367	495	4	104	108	48	8.8	26	19.4
Total	2189	539	513	1406	1919	11	403	414	270	12.3	125	23.2

¹ All values are in terms of mg/rat, except as otherwise noted.

period and Ca = milligrams of calcium retained for a 4-week period.

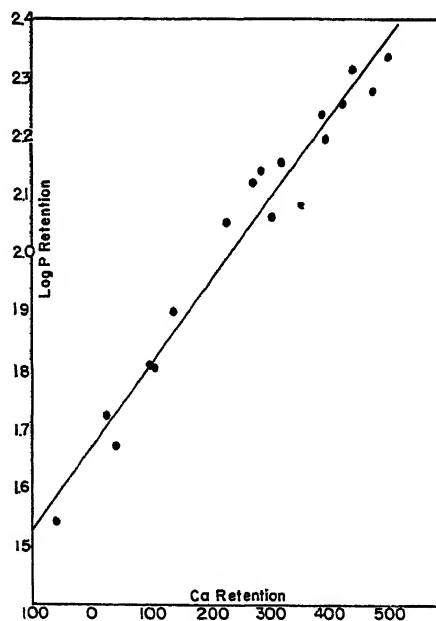


Fig. 1 Ca vs. log P retentions of rats on Steenbock-Black diet. Equation of solid line is $\log P = 1.37 \times 10^{-3} \text{ Ca} + 1.67$. Values shown are in terms of milligrams retained per rat for a 4-week period.

It might also be noted that in no case from among the 72 weekly phosphorus retention values for the 18 rats on the rachitic diet was a phosphorus loss observed, although in a few cases (9 out of 72) a weekly loss of calcium was noted; three of these 9 involved one rat. It appears, insofar as this study is concerned, that phosphorus can be retained without calcium but that the latter is not retained without the former. It may also be said that for the rats on the Steenbock-Black diet retention was poorest on those diets having the greatest Ca to P ratios.

SUMMARY AND CONCLUSIONS

The hypothesis originally offered to explain the prevention of rickets in rats on a standard rickets-producing diet when the acid salts of citric and tartaric acids are added, was that an acid-base factor was involved (Hamilton and Schwartz, '33; Shohl, '37). These salts produce an acid medium when in the gastrointestinal tract, thus increasing the solubility of the calcium and hence its absorption. The alkaline ash resulting from the metabolism of these salts provides an environment favorable for bone formation. Shohl ('37), in addition to considering the acid-base factor, suggested the possibility that complex formation might also be involved.

In the present investigation, designed to evaluate the effect of complex formation on calcium retention, the factors of relative acidity and alkalinity were not apparently involved, since the complex-former used and its control material would appear to be neutral in character both in the gastrointestinal tract and upon metabolism in the body. The enhanced calcium retention produced by Am.P. can therefore result insofar as is known only from its complex-forming properties. This bears out the hypothesis of previous investigators that complex formation may be an important factor in calcium metabolism.

Summaries are given of data on the Ca and P retentions of 18 rats maintained on the Steenbock-Black diet, and on the relationship between the Ca and P retentions.

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THE EFFECT OF MIXED TOCOPHEROLS ON MILK AND BUTTERFAT PRODUCTION OF THE DAIRY COW¹

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Harris et al. ('47) reported a surprising response of farm milch cows in butterfat and total milk production when daily supplements of 1 gm of mixed tocopherols were fed. An effort has been made to confirm these results and the experimental findings are reported herewith.

EXPERIMENTAL

The plan of this experiment was designed to make use of a farm herd of pure-bred Holstein-Friesian cattle. Conditions on this farm were average and typical of the management practices of the area. Cattle were handled under the so-called "loose-run" system. The herd was above average in production and had for the previous three years on Official Herd Improvement Registry produced an average in excess of 400 lb. of butterfat per cow. The care, management and feeding of these cattle were directly under the supervision of one of the present authors (P.H.P.). Daily records of milk production were kept, but in all data reported here the HIR monthly

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tests and their estimation of the average daily milk production, butterfat yield and per cent fat were used. This placed the sampling and testing in the hands of disinterested parties, and since these records have official acceptance they seemed to be sufficiently reliable for our use.

The cows were divided into two lots of 9 cows each on the basis of age, stage of lactation, calving record and potential milk production. In most instances daughters of the same bull were divided between lots. Since this herd is a line-bred herd, the variable due to breeding was somewhat reduced. One lot served as the control (Lot I) and the other (Lot II) was fed the basal ration plus 21.3 gm of a mixed tocopherol preparation, which furnished 1 gm of mixed tocopherols per cow per day (the level fed by Harris et al., '47). This was fed once daily by incorporation with the grain mixture in the evening feed. No difficulty was experienced in getting the cows to eat the mixture.

The ration used was composed of good quality corn silage ensiled in the early dough-to-dough stage of maturity; alfalfa-brome grass hay, field-cured and field-chopped at a theoretical length of 1.75 inches; and a 16% grain ration. The hay was estimated to be composed of 40% alfalfa and 60% brome for the first cutting and 75% alfalfa and 25% brome for the second cutting. The hay was cut at the recommended half-bloom stage and since it was "layered" in the barn it was possible to feed a mixture of first and second cuttings in the ratio of 3:1. The hay was fed ad libitum, mixed in approximately this ratio. The grain mixture was composed of the following, in pounds: ground wheat 400, ground oats 400, dried brewers' grain 200, soybean oil meal 100, wheat bran 100, bone meal 20, and trace mineralized salt 20. The tocopherol content of the ration concentrates was determined by the method of Hines and Mattill ('43). The hay averaged 42 mg, the silage 7 mg and the grain mixture 14 mg of tocopherol per pound on an air-dried, uncorrected-moisture basis. The estimation of the daily tocopherol ingestion through the ration was approximately 1 gm per cow per day (0.99 gm), a figure closely

approximating that found occurring naturally in the ration used by Harris et al. ('47).

Two distinct experimental periods were used. Experiment I was set up with the two lots of 9 cows each, and in it we studied the effect of the incorporation of the mixed tocopherol, as indicated above. Experiment II, beginning at the close of Experiment I, was used to test the effect of mixing the tocopherols directly with the whole grains of the grain mixture at this time of grinding. In Experiment II all cows in both lots were fed the grain mixture with the mixed tocopherols incorporated. The experimental period for Experiment I lasted from December 10, 1947, through March 9, 1948, while Experiment II began March 10, 1948, and carried through for 6 weeks. The second experiment was designed to determine whether an antioxidation effect was present when the mixed tocopherols were placed with the grain mixture before grinding. Ground whole grains are known to heat after grinding and it was thought that the tocopherols might prevent undue oxidation.

RESULTS

When the results were compiled, it was found that three cows had to be eliminated from the records because of premature "drying off" or other abnormal conditions. The data which show the effect of the feeding of mixed tocopherols upon milk and butterfat production are given in table 1.

TABLE 1

Effect upon milk and butterfat production of feeding 1 gm daily of mixed tocopherols

LOT	NO. COWS	AVERAGE DAILY MILK YIELD BY MONTHS				AVERAGE DAILY BUTTERFAT				AVERAGE ESTIMATED BUTTERFAT YIELD BY MONTHS			
		Prior (Nov.)		After		Prior (Nov.)		After		Prior (Nov.)		After	
		30 days	Dec.	Jan.	Feb.	30 days	Dec.	Jan.	Feb.	30 days	Dec.	Jan.	Feb.
Control Toco- pherol- fed	7	lb. 45.8	lb. 42.4	lb. 42.4	lb. 38.1	% 3.5	% 3.5	% 3.3	% 3.7	lb. 48.8	lb. 45.5	lb. 43.7	lb. 40.7
	8	45.4	41.9	39.4	33.4	3.5	3.5	3.6	3.5	47.9	43.7	44.3	34.7

It is quite evident that the addition of 1 gm of mixed tocopherols daily to the ration of these cows did not increase milk production or the total butterfat production per month. There was some variation in the per cent of butterfat but this was well within the normal range, and there was no difference between groups. Paired as these cows were, there was a remarkable similarity in the performances of the two lots.

Table 2 shows the milk and butterfat yields for all cows during Experiment II. Again, there was no increase in either milk or butterfat production for this 6-week period and the per cent of fat was remarkably constant.

TABLE 2

Effect on both lots of cows of mixing tocopherols with grain mixture at the time of grinding

LOT	NO. COWS	AVERAGE DAILY MILK YIELD BY MONTHS		AVERAGE DAILY BUTTERFAT		AVERAGE ESTIMATED BUTTERFAT YIELD BY MONTHS	
		March	April	March	April	March	April
Control	4	lb. 38.1	lb. 34.0	% 3.4	% 3.5	lb. 37.8	lb. 35.2
Tocopherol-fed	7	34.6	30.8	3.4	3.5	36.6	32.5

In order to make certain that the stage of lactation or that age did not affect these results, the data were assembled by paired groups, as is shown in table 3. When the cows were paired on the basis of the stage of lactation there was no difference in milk production between the controls and the tocopherol-fed lot. When paired by age, either at the first calf heifer stage or at the mature cow level, the differences again were not significant. The drop in production was very similar in both groups and in most cases the controls produced as well or better than the cows fed the tocopherol preparations. Any differences appearing in the data are due largely to individual variations during the period studied. If the butterfat production and per cent of butterfat are examined on the basis of pairing by stage of lactation or by

age, as in the case of milk production, no significant differences are noted (table 4). At the end of the first experimental period complete blood analyses for vitamin A and carotene

TABLE 3

Effect of feeding tocopherols on the average daily milk production by months

LOT	NO. OF COWS	AVERAGE PRIOR 30 DAYS	EXPERIMENTAL PERIOD (MONTH)		
			1	2	3
		lb.	lb.	lb.	lb.
Cows paired by stage of lactation (8-10 weeks)					
Control	6	46.8	44.0	44.0	41.0
Tocopherol-fed	6	45.7	44.4	42.3	37.9
Cows paired by age (2-year olds)					
Control	3	40.5	39.7	39.6	38.1
Tocopherol-fed	2	37.2	37.0	34.7	32.6
Cows paired by age (4-5 years)					
Control	3	53.0	48.2	48.4	43.9
Tocopherol-fed	4	49.9	45.5	46.0	40.5

TABLE 4

Effect of tocopherol feeding on the average monthly butterfat production and average fat content of milk

LOT	NO. OF COWS	AVERAGE MONTHLY BUTTERFAT PRODUCTION				AVERAGE FAT CONTENT OF MILK			
		Ave. prior 30 days	Experimental period in months:			Ave. prior 30 days	Experimental period in months:		
			1	2	3		1	2	3
			lb.	lb.	lb.		%	%	%
Cows paired by stage of lactation (8-10 weeks)									
Control	6	50.1	47.5	44.9	43.1	3.6	3.5	3.3	3.7
Tocopherol-fed	6	49.6	44.6	47.0	39.8	3.6	3.5	3.5	3.6
Cows paired by age (2 years old)									
Control	3	42.3	41.5	38.8	39.0	3.5	3.5	3.2	3.6
Tocopherol-fed	2	37.9	39.1	35.0	35.0	3.4	3.4	3.5	3.8
Cows paired by age (4 to 5 years old)									
Control	3	54.8	53.6	50.6	47.3	3.8	3.6	3.4	3.7
Tocopherol-fed	4	55.4	47.3	51.8	42.5	3.7	3.4	3.6	3.6

were made on the entire herd. The control group averaged 25.2 $\mu\text{g } \%$, as compared with the tocopherol-fed group with 21.4 $\mu\text{g } \%$. Both of these groups were well above the marginal level of 15 $\mu\text{g } \%$ and were, therefore, adequately supplied with vitamin A. The carotene content was ample in both instances.

A pooled sample of blood, representing 0.2 ml of blood plasma for each cow in the lot, was taken at the close of the first experiment and the tocopherols estimated according to the method of Mayer and Sobotka ('42). The control group averaged 0.70 mg % tocopherol, as compared to the tocopherol-fed lot with 0.68 mg %. Apparently the feeding of mixed tocopherols did not raise the tocopherol content of the blood of these animals.

It is apparent from these data that we were unable to confirm the work of Harris et al. ('47), in that we could not produce an increase in either milk production, butterfat production or the per cent of butterfat with the ration used. It is possible that the character of the ration, particularly the grain portion, differs sufficiently from that used by Harris et al. to account for the difference in results.

SUMMARY AND CONCLUSIONS

An experiment was set up in which a preparation containing 1 gm of mixed tocopherols was fed to milch cows to determine its effect upon milk and butterfat production. The results indicate definitely that the inclusion in the ration of 1 gm of mixed tocopherols per cow daily does not influence the milk or butterfat yield, and neither does it affect the per cent of butterfat present in the milk. These data do not substantiate the work of Harris et al. ('47), at least when the mixed tocopherols are fed under the conditions prevailing during the present experiment.

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NITROGEN BALANCE INDEX AND SPECIFIC DYNAMIC ACTION IN RATS RECEIVING AMINO ACID MIXTURES LOW IN ISOLEUCINE, METHIONINE OR VALINE^{1,2}

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ONE FIGURE

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The quality of a diet may be measured in terms of efficiency of utilization of energy, nitrogen or some other nutrient. Biological value and nitrogen balance index are measures of efficiency of utilization of nitrogen, and are preferable to body weight observations for the reasons given by Mitchell ('44). The efficiency of utilization of energy of the diet can be expressed as the "net availability of metabolizable energy" (NAME) (Mitchell, '34):

$$\text{NAME} = 1 - \frac{\text{SDA}}{\text{ME}} \quad (1)$$

SDA is specific dynamic action and ME is metabolizable energy. Since ME is that portion of the total energy intake which is not lost in the excreta, and SDA is the wasted portion

¹ Many of the data of this paper are taken from a thesis submitted by Joseph T. Anderson ('47) to the faculty of the Graduate School of the University of Rochester in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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of ME, the above expression gives the fraction available for basal metabolic rate, growth, activity and other productive processes.

In a series of diets fed so as to give equal ME, the one having the highest NAME will produce the lowest SDA. It follows, therefore, that among diets of equal metabolizable energy, specific dynamic action is an inverse measure of efficiency of energy utilization. Mitchell ('34) stated that the best diet for energy retention is the one which yields the highest NAME. Hamilton ('39) and Forbes and Swift ('44) demonstrated that there is an optimum dietary content of vitamins, minerals, protein and fat, and that diets which are not the optimum in these respects are not utilized by the animal at maximum efficiency.

Gross deficiency of any essential amino acid evidently causes a considerable decrease in efficiency of utilization of both energy and nitrogen, since animals may continue to eat such a deficient diet yet lose weight drastically. It may be expected, then, that a diet which is only mildly deficient in one essential amino acid will show a small decrease in efficiency of both nitrogen and energy utilization.

The purpose of our investigation, the first portion of which is described below, was to determine quantitatively by means of N balance index and specific dynamic action the influence of diets, mildly deficient in one essential amino acid, upon the efficiency of utilization of nitrogen and energy for maintenance of the mature rat.

METHODS

Nitrogen metabolism

Each group of experimental animals consisted of 10 to 12 mature male albino rats. The maximum differences in weight and age were 50 gm and 15 days, respectively. A single group of rats served for three to 5 consecutive experiments. Series 20 rats were 34 weeks old and series 40 rats 24 weeks old at the time of feeding the first amino acid mixture diet. The sequence of feedings in a single cycle were: (1) two or three

weeks of recovery diet; (2) one week of N-free diet; (3) one week of amino acid mixture diet; (4) one and one-half days of fasting. This cycle was repeated as often as seemed desirable. The arrangement of the diets was intended to bring the average body weight of the animals to the same value at the beginning of each amino acid diet period. The N-free period served to deplete the protein stores of the rats to a reproducible extent before each amino acid feeding.

TABLE 1
Composition of diets

COMPONENT	COMPLETE AMINO ACID DIET	N-FREE DIET	RECOVERY DIET
<i>per 100 gm diet</i> ¹			
"Complete" amino acid mixture	4.19 gm		
Dried whole egg ²			20.1 gm
Sucrose	65.5 gm	69.7 gm	58.3 gm
Cottonseed oil (Wesson)	17.4 gm	17.4 gm	8.75 gm
SiO ₂ ³ or Fe ₂ O ₃ for marker	2.3 gm	2.3 gm	2.3 gm
Salt mixture (Wesson, '32)	4.5 gm	4.5 gm	4.5 gm
Cellu flour	5.8 gm	5.8 gm	5.8 gm
N from amino acids or egg	0.53 gm	0 gm	1.53 gm
Estimated physiological heat value	437 Cal.	437 Cal.	424 Cal.

¹ One hundred grams of each diet contained 0.92 mg thiamine hydrochloride, 0.92 mg pyridoxine hydrochloride, 1.15 mg methyl naphthoquinone, 1.84 mg riboflavin, 4.6 mg niacin amide, 5.1 mg calcium pantothenate, 4.6 mg alpha tocopherol, 112 mg Navitol containing 65,000 A and 13,000 D units per gram, 115 mg choline chloride and, in the series 40 experiments, also 0.06 mg biotin, 0.06 mg folic acid and 23 mg inositol.

² The dried whole egg contained 7.60% N and 40.5% fat.

³ Isco blanc rouge, Innis Speiden and Co., New York.

The composition of the diets is given in tables 1 and 2. Series 20 rats were fed material furnishing 37.7 Cal./day/rat of estimated physiological heat value (metabolizable energy), the quantity being the same for every rat and in every period. Metabolizable energy was estimated as 4, 9 and 4 Cal. per gram of protein, fat and carbohydrate, respectively. Series 40 rats were larger and were allowed 40.1 Cal./day/rat. The

feeding at the specified caloric intake of the recovery diet, which contained 9.6% protein, permitted a slow gain in weight.

The N intake from the amino acid diets was a little less than the N excretion of the rats on the N-free diet. This resulted in a negative N balance in all amino acid diet periods which, according to the observations of Allison and Anderson ('45) with dogs, insured a maximum N balance index.

TABLE 2
Amino acid composition of egg and of diets

AMINO ACID	WHOLE EGG		"COMPLETE" AMINO ACID MIXTURE		
	Dried whole egg ¹	Acetone-ether insoluble ²	Natural isomer present	Isomer used	Total amino-acid used
<i>mg amino acid N/gm total N</i>					
Arginine	128	195	192.3	L	192.3
Histidine	36	62	49.4	L	49.4
Isoleucine	53	47	45.7	DL	91.4
Leucine	61	65	63.4	L	63.4
Lysine	86	94	81.8	L	81.8
Methionine	24	23	22.7	DL	45.4
Phenylalanine	33	32	32.0	DL	63.9
Threonine	36	36	35.8	DL	71.7
Tryptophane	13	14	14.2	DL	28.3
Valine	55	54	53.9	DL	107.8
Glutamic acid			204.5	L	204.5
Total N	1000	1000			1000

¹ Mitchell and Block ('46).

² Edwards et al. ('46).

Table 2 gives the composition of the "complete" amino acid mixture for comparison of its essential amino acids with two analyses of whole egg protein. The amount of each natural form of amino acid included in the "complete" amino acid mixture, except for some discrepancy in the cases of histidine and lysine, is close to that in the analysis reported by Edwards et al. ('46). Glutamic acid was added to make the total N the same as in egg. This mixture, therefore, resembled egg protein very closely in respect to its content of the natural isomers of the essential amino acids. The non-essential amino acids

in egg were replaced in the mixture by the unnatural forms plus glutamic acid.

Another amino acid mixture called the "low-isoleucine" mixture differed from that shown in table 2 in that the amount of DL-isoleucine was reduced to one-third. The total N content was kept constant by the addition of more glutamic acid. In analogous fashion "low-methionine" and "low-valine" mixtures were prepared. A "complete" amino acid mixture was used also in which the glutamic acid was replaced by glycine containing the same amount of N. Since the physiological heat values of all the amino acid mixtures were close to that of sucrose, any change in the total weight of amino acids used per 100 gm of diet was compensated for by an equal and opposite change in weight of sucrose.

Since some rats failed voluntarily to eat the prescribed quantity of N-free diet for 7 days, this diet, as well as the amino acid diets, was fed every 12 hours by stomach tube. The dry diet was mixed thoroughly with about 35% of water and injected from a pump through a no. 8 French-size Wishart catheter. The brass pump used was a gasoline blowtorch air pump in which the piston was sealed by a leather washer. Stops placed on the piston shaft limited its travel to give the desired volume. The pump, the catheter and a storage reservoir were connected to a three-way metal stopcock in such a way that the syrupy diet could be forced by air pressure into the pump and subsequently discharged from the pump through the catheter.

Nitrogen intake was determined by analysis of portions of diet exactly like those given the rats and delivered from the same catheter. The feces for each diet were separated by giving Fe_2O_3 -marked diet at the first feeding of each dietary period, and by giving a mixture of Fe_2O_3 , agar and cellulose flour by stomach tube on the first morning of the fast. Urine was collected at the end of the third, 5th and 7th days of the N-free and amino acid diet periods by washing out the cage with hot water a few minutes after feeding the rat. Nitrogen

determinations were carried out by the micro-Kjeldahl method of Miller and Houghton ('45).

Energy metabolism

The heat production of the rats was measured during the greatest possible part of the daylight half of the last day on each amino acid diet and again for a similar time period 48 hours later, after a 36-hour fast. Each rat was placed in a body size screen cage, so small that the rat could not turn around. Each cage rested in a stainless steel box which collected the urine passed during the test. Six cages were placed together in a battery jar, which was sealed and made part of a Haldane small animal metabolism train. Two CO₂ absorbing units were provided. One of these was used only when the rats were quiet, and the other for the remainder of the time.

After equilibration with a stream of dry CO₂-free air at 10 l/min., the battery jar containing the rats was weighed at about 10:30 A.M. and then connected so that all the H₂O and CO₂ given off by the rats was collected until about 8:30 P.M. The rate of air flow was 10 l/min. at all times. The rats were observed for extended periods; when they were all quiet the air stream was changed to the "quiet" CO₂ absorber, but it was returned to the "active" CO₂ absorber as soon as any one of them had been active for one and one-half to two minutes. Approximately one hour of quiet period was accumulated during each third of the 10-hour total period. The average R.Q. for the entire period was computed from the total CO₂ production and O₂ consumption. This R.Q. together with the quiet period CO₂ production and the urinary N excretion rate was used to compute the resting heat production rate. A similar method was used by Forbes and Swift ('44).

Since our purpose was to determine the influence of the amino acid composition of the diet on the heat production, all the other factors known to change heat production were controlled.

The environmental temperature during the test was held between 28 and 31°C. and between 26 and 29°C. during the remainder of the experiment. Activity cycles which are known to cause metabolic rate variations were regulated by: (1) using males, which are free from estrous cycles; (2) regularly keeping the room light from 9 A.M. to 9 P.M. and dark from 9 P.M. to 9 A.M.; and (3) feeding the animals daily at 9 A.M. and 9 P.M. The body size was kept substantially constant. The caloric intake was constant from day to day except during the fast. The size of the meal administered at the beginning of the test was always the same. Although activity during the heat production test was controlled as described above, this factor was probably the most important source of variability in the heat production values.

RESULTS

Nitrogen metabolism

Three experiments were carried out with the rats of series 20, testing, in the order named (1) the "complete" amino acid mixture, (2) the low isoleucine mixture, and (3) the "complete" amino acid mixture. The average N balance data are given in table 3. The urine N values are from the last 4 days of each 7-day diet period. These gave a smaller standard deviation of N balance index than urinary N from the last two days only, and there was no consistent difference between the average urinary N of the 6th and 7th days and the average urinary N of the 4th and 5th days.

Nitrogen balance index was defined by Allison and Anderson ('45). All experimentally-determined biological values in which endogenous nitrogen excretion is estimated on the basis of total nitrogen excretion on a nitrogen-free diet are really nitrogen balance indices. We accept the suggestion of Allison, Seeley, Brown and Anderson ('46) that the term "biological value" be reserved for the fraction of absorbed nitrogen which is retained according to computations based

TABLE 3
Average nitrogen balance data for rats receiving amino acid mixture diets

EXPERIMENT NUMBER NUMBER OF RATS BODY WEIGHT ¹ (KG) METABOLIC BODY SIZE (KG ^{3/4}) BODY SURFACE ² (M ²)	SERIES 20				SERIES 40			
	I	II	III		I	II	III	IV
	10	9	8		12	12	12	17
	0.212	0.212	0.221		0.230	0.232	0.231	0.231
	0.312	0.312	0.322		0.322	0.334	0.333	0.336
	0.0314	0.0314	0.0321		0.0328	0.0350	0.0328	0.0351
N balance data (mg N/day/kg ^{3/4})								
"N-free" period								
NI ¹ (Nitrogen intake)	5	4	5		10	6	6	6
FN ¹ (Feces nitrogen)	45	42	38		40	39	38	41
UN ¹ (Urine nitrogen)	194	203	164		144	140	139	145
Amino acid period								
Amino acid mixture						Comp. +	Low meth-	Low
NI (Nitrogen intake)	Complete	leucine	Complete	Complete	Complete	glycine	ionine	valine
FN (Feces nitrogen)	152	151	145	156	146	141	141	142
UN (Urine nitrogen)	41	39	39	37	34	34	34	37
	127	177	122	131	117	117	202	128
NE ₀ (N excretion at zero NI)	242	246	203	185	180	180	175	196
Stand. dev. of mean NE ₀	6	8	8	4	10	10	5	9
AN ₀ (Absorbed N to give equilibrium)								
Stand. dev. of mean AN ₀	168	212	156	171	155	155	323	163
	4	6	3	4	3	3	15	4
P ₁ for AN ₀ ²	< 0.0001	0.02	0.02		0.004	< 0.0001	0.18	0.04
P ₂ for AN ₀ ²	< 0.0001				0.4	< 0.0001	0.5	
D (True digestibility)								
	1.03	1.02	0.99	1.02	1.03	1.03	1.03	1.02
K (N balance index of absorbed N)								
Stand. dev. of mean K	1.44	1.17	1.30	1.09	1.16	1.16	1.18	1.15
	0.04	0.05	0.04	0.02	0.06	0.06	0.04	0.02
P ₁ for K ²	0.001	0.04	0.04		0.30	< 0.0001	0.09	0.09
P ₂ for K ²	0.08				0.9	< 0.0001	0.5	

¹ Average body weight at end of amino acid diet period.

² Formula (2) of Lee ('29): (m²) = 0.001254 (gm)^{0.75}.

Probability that the differences between mean values might have arisen due to chance only, computed from the Student "t" value (Kenney, '39). P₁ is for comparison of the period indicated with the preceding "complete" amino acid period. P₂ is for comparison of the period indicated with the following "complete" amino acid period.

on the true endogenous N excretion. Biological value can be obtained from the expression

$$NB = (BV) (AN) - EN \quad (2)$$

in which EN is the true endogenous N excretion, AN is absorbed N, NB is N balance and BV is biological value. The nitrogen balance index is K in the following equation:

$$NB = K (AN) - NE_0 \quad (3)$$

in which NE_0 is N excretion on a N-free diet. Since true endogenous N probably cannot be determined, neither can biological value as defined by Allison et al. The function obtained experimentally is N balance index.

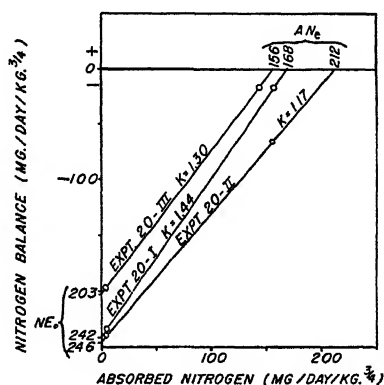


Fig. 1 N balance experiments with series 20 rats fed amino acid mixtures. The low isoleucine diet of experiment 20-II gives a low N balance index (slope of the line = K) and a high requirement of absorbed N for equilibrium (AN_0) compared with the complete diet of experiments 20-I and 20-III.

In figure 1 the results of the three experiments of series 20 are presented as plots of N balance against absorbed N. Each experiment yields two points, the lower one from the N-free diet and the upper one from the amino acid diet. Since Allison and Anderson ('45) have shown that all such points fall on a straight line conforming to the relationship expressed in equation (3), lines have been drawn through each pair of points. The slope of each line is the N balance index of ab-

sorbed N, which is K in equation (3). The lower intercept with opposite sign is NE_0 , the N excretion at zero N intake. The upper intercept, AN_0 , is the absorbed N necessary to give N equilibrium or the maintenance N requirement for the amino acid mixture used. The relationship among these quantities is obtained by making NB equal to 0 in equation (3).

$$K = \frac{NE_0}{AN_0} \quad (4)$$

The AN_0 value for a diet is determined chiefly by the N balance data of the amino acid diet period and is only slightly influenced by the N-free period, as can be appreciated from the lines plotted in figure 1. K, on the other hand, is dependent equally on the data from both periods. Thus, although AN_0 and K are both measures of the quality of the diet for promotion of N retention and are both computed from the same experimental data, they may be expected to give somewhat different indications of quality, since the relative weights of the data in the two computations are quite different.

N balance index (K) does not depend on body size. AN_0 and NE_0 are directly proportional to metabolic body size, which, according to Kleiber ('47), is well expressed by the three-quarters power of body weight. Unless some qualitative difference in N metabolism exists, N requirements for N equilibrium expressed as mg N/day/kg^{3/4} should be nearly the same for large and small rats, as well as for various species.

The N balance index for the low isoleucine diet was 1.17, as compared with 1.44 and 1.30 for the "complete" amino acid mixture diet. The difference is highly significant in one case and of doubtful significance in the other, as indicated by probabilities of 0.001 and 0.08. The AN_0 for the low isoleucine diet was 212 mg N/day/kg^{3/4}, as compared with 168 and 156 for the "complete" amino acid mixture diet. These differences are both highly significant. It is therefore concluded that the low isoleucine amino acid mixture is utilized by the

rat for maintenance of N equilibrium less efficiently than the "complete" amino acid mixture.

An abnormal appearance of the eyes of the rats began to appear in experiment 20-II and was quite evident in experiment 20-III, at which time it was diagnosed as "spectacle-eye" due to biotin deficiency. At the end of experiment 20-III the rats were placed on the recovery diet supplemented with 5 μ g of biotin per rat per day, and at the end of three weeks the eyes had definitely improved and hair had begun to grow in the previously bald areas. Apparently a diet containing dried whole egg needs supplementation with biotin. Although it is possible that the biotin deficiency may have influenced the N balance and energy metabolism, there is no indication of it in the data.

The series 40 rats, which unfortunately came from a different colony, were used to compare the "complete" amino acid mixture with three other mixtures. The only difference in the dietary amino acid mixture of period 40-II was the isonitrogenous substitution of glycine for glutamic acid. The "complete with glycine" mixture gave a N balance index (K) which was not significantly different from the one obtained in either the preceding or following trials with the "complete" amino acid mixture containing glutamic acid. Comparison of AN_e values indicates a significant difference between 40-I and 40-II but not between 40-II and 40-V. In spite of one discordant result in 4, it is concluded that substituting glycine for glutamic acid in amounts containing equal N causes no change in efficiency of utilization of N of the amino acid mixture.

A low methionine amino acid mixture in which 1.51% of the N was provided by DL-methionine was fed in experiment 40-III. The N balance index was 0.55, which is a most striking decrease from the values of 1.09 and 1.15 for the "complete" amino acid mixture. The amount of absorbed N necessary to give equilibrium when feeding the low methionine amino acid mixture was found by extrapolation to be 323 mg N/day/kg^{3/4}, which is markedly higher than the 171 and

159 mg computed for the "complete" amino acid mixture. The low N balance index and high AN_e both indicate that the amount of methionine in the low methionine diet is so small as to depress the efficiency of utilization of N of the amino acid mixture.

In experiment 40-IV the valine was reduced to one-third of the amount in the "complete" amino acid mixture, so that 3.59% of the N was supplied by DL-valine. There was no significant difference between the N balance index and AN_e in this period and the values in the preceding or following "complete" amino acid periods. It is concluded that the low valine amino acid mixture provides enough valine to support full efficiency of N utilization.

Energy metabolism

In connection with each amino acid diet the resting post-cibal metabolic rate and the resting fasting metabolic rate of the rats were determined as described above. The metabolic rate values are listed in table 4 and are to be understood as values obtained during about three hours of observed rest distributed fairly uniformly through the light half of a day and computed on a 24-hour basis. The two values which are subtracted to give the specific dynamic action were determined on the same individuals, with a time interval of only 48 hours between the two tests. The results are expressed as Cal./day/rat, since each animal in a given series received the same amount of diet regardless of individual or time variations in body weight. The SDA values in each series of experiments represent, therefore, the average effect of equal amounts of estimated metabolizable energy in rats of varying body weights.

As is indicated in table 4, the SDA of the low isoleucine diet, 6.0 Cal./day/rat, was found to be significantly greater than the 2.6 and 3.1 Cal./day/rat for the "complete" amino acid diet. This indicates that decreasing to one-third the isoleucine in the "complete" amino acid mixture caused a decrease in efficiency of utilization of metabolizable energy, as well as

TABLE 4
Average resting energy metabolism data for rats receiving amino acid mixture diets

EXPERIMENT NUMBER	SERIES 20			SERIES 40				
	I	II	III	I	II	III	IV	V
Diet	Complete	Low-iso-leucine	Complete	Complete	Comp. + glycine	Low methionine	Low valine	Complete
Estimated metabolizable energy (Cal./day/rat)	37.7	37.7	37.7	40.1	40.1	40.1	40.1	40.1
Number of rats	10	10	8	12	12	12	11	11
Body wt. ¹ (kg)	0.212	0.212	0.221	0.230	0.232	0.231	0.231	0.234
Respiratory quotients:								
Receiving diet	0.96	0.93	0.95	0.97	0.96	0.95	0.97	0.94
After 36-hr. fast	0.71	0.73	0.73	0.73	0.71	0.73	0.72	0.72
Metabolic rates (Cal./day/rat):								
Receiving diet	23.1	27.7	24.7	27.2	25.8	26.6	27.6	26.4
After 36-hr. fast	20.5	21.7	21.6	23.7	24.4	24.4	23.4	24.1
SDA	2.6	6.0	3.1	3.5	1.4	2.2	4.2	2.3
Standard dev. ²								
of mean SDA	0.8	0.8	0.8	1.0	1.0	1.0	1.0	1.0
P ₁ ³		0.006	0.7		0.15	0.4	0.6	0.4
P ₂ ³		0.02			0.6	1.0	0.2	

¹ Mean body weight at end of amino acid diet period.

² A single estimate of the standard deviation of a mean SDA was computed for each series from all the differences found on various days between the average metabolic rates of the two matched groups in the two metabolism units.

³ Probabilities that the differences between mean SDA values occurred by chance. P₁ is for comparison with the preceding "complete" amino acid period and P₂ is for comparison with the following "complete" amino acid period.

a decrease in efficiency of utilization of absorbed N. No significant alteration in SDA appeared with other changes in the amino acid composition of the diet, although by analogy with the N balance data the low methionine diet should have caused an increase in SDA.

DISCUSSION

Body N-sparing effect

The data shown in table 3 indicate that in every experiment except that involving the low methionine diet the urinary N excretion was decreased when an amino acid mixture was introduced into a N-free diet. This fact accounts for a N balance index greater than unity. As pointed out by Allison, Seeley, Brown and Anderson ('46), the N balance index must be higher than the biological value and the N excretion at zero N intake (NE_0) must be greater than the true endogenous N excretion (EN) in such a circumstance.

Nielson, Gerber and Corley ('39) found that the incorporation of L-cystine into the N-free diet of dogs caused the urinary N to be decreased on the day of the treatment and for a few days thereafter. They also observed a similar response after administering a mixture containing all the essential amino acids except threonine. Miller ('44) observed that the addition of methionine or cystine to a very low protein diet caused a 30 to 45% reduction in N excretion. Allison, Seeley, Brown and Anderson ('46) and Allison, Anderson and Seeley ('47) demonstrated that feeding methionine, methionine-supplemented proteins and unsupplemented egg white protein diminished the urinary N in the dog and that the effect was more marked the longer the preliminary period of protein-free feeding. Urinary N in the rat is decreased by supplementing a N-free diet with any of the following: methionine, cystine, arginine, isoleucine, histidine, choline or a mixture of the 10 essential amino acids (Brush, Willman and Swanson, '47). According to Johnson, Deuel, Morehouse and Mehl ('47) and Cox et al. ('47), methionine feeding does not reduce urinary N in man even after protein depletion.

Minimum urinary N

Evidently the urinary N excretion of the rat on a N-free diet is altered considerably by variations in protein depletion or by the addition to the diet of small amounts of certain amino acids. In the data shown in table 3 the NE_o values for a single diet are observed to differ among experiments much more than do the AN_o values. Since $K = NE_o/AN_o$, variability in NE_o is transmitted directly to K . In comparing experiments 20-I, 20-III, 40-I, 40-II, 40-IV and 40-V, AN_o is seen to be a much more consistent attribute of the diet than K . It is suggested that the use of urinary N excretion on a N-free diet in computing N balance index in rats leads to difficulties in two ways: (1) N balance indices are higher than true biological values and (2) variability in N balance index occurs among groups of animals and between dietary periods with the same group. An alternative procedure is to feed the test protein or amino acid mixture at two levels.

The mean urinary N excretion in 6 experiments with the N-free diet was 157 ± 8 mg/day/kg^{3/4}. The addition of an adequate amino acid mixture to this basic, N-free diet resulted in a highly significant diminution in urinary N excretion. In the same 6 experiments in which the "complete" amino acid mixture or its equivalent was fed, the urinary N excretion was reduced to 125 ± 2 mg/day/kg^{3/4}. It seems likely that refinements in the amino acid mixture may reduce this further so that the present value should therefore not be considered the absolute minimum. If related to the energy metabolism, it represents an excretion of 1.79 mg N per basal Calorie, which is lower than previously published values for the rat (Smuts, '35: 1.988 mg; Bricker and Mitchell, '47: 2.16 mg) and somewhat higher than recently reported values for man (Hawley, Murlin, Nasset and Szymanski, '48: 1.19 mg for men and 1.32 mg for women). It is interesting also that in 7 of the 8 experiments (table 3), feeding amino acid mixtures reduced the fecal N as compared with the corresponding N-free periods.

SUMMARY AND CONCLUSIONS

Simultaneous nitrogen balance and energy metabolism studies were conducted on adult rats receiving amino acid mixtures in a diet otherwise very low in nitrogen. Each amino acid diet period was preceded by a 7-day depletion period in which an isocaloric N-free diet was fed. The diets were administered by stomach tube twice daily in order to insure equal intake by every animal every day. Resting energy metabolism determinations were made during a 10-hour period on the last day of the amino acid diet and again two days later after a fast of 36 hours. The difference between these rates was taken as representing the specific dynamic action of the diet.

When a "complete" amino acid mixture simulating whole egg protein was fed the N balance index was invariably greater than unity. The N requirement for maintenance on this amino acid mixture averaged 162 mg N/day/kg^{3/4}. In 6 experiments with two groups of rats the maintenance N requirement proved a more consistent characteristic of the amino acid mixture than the N balance index.

Reducing the DL-isoleucine to one-third the quantity in the "complete" amino acid mixture caused a decrease in N balance index, an increase in maintenance N requirement to 212 mg N/day/kg^{3/4}, and an increase in specific dynamic action of 3 Cal./day/rat. A similar reduction in DL-methionine caused a decrease of N balance index to about half and an increase of maintenance N requirement to 323 mg N/day/kg^{3/4} but no significant change in specific dynamic action. Reduction of DL-valine to one-third the quantity in the "complete" amino acid mixture caused no significant change in either N utilization or energy metabolism. Substituting glycine for glutamic acid caused no significant change in N balance index, maintenance N requirement or specific dynamic action.

Reducing the quantity of an essential amino acid will, if carried far enough, lead to decreased efficiency of utilization of N (decreased N balance index and increased maintenance N requirement), and probably to a parallel decrease in effi-

ciency of energy utilization (increased specific dynamic action). Such a decrease in efficiency of N utilization was found when either isoleucine or methionine was reduced to one-third. The corresponding decrease in efficiency of energy utilization was found only in the case of the low isoleucine diet.

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THE PLACENTAL AND MAMMARY TRANSFER OF TOCOPHEROLS (VITAMIN E) IN SHEEP, GOATS AND SWINE

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Willman et al. ('45, '46) have shown that when ewes are fed a ration consisting of legume hay and red kidney beans during gestation and lactation, a high percentage of the suckling lambs develop muscle dystrophy ("stiff-lamb disease"). The pathological picture appears to be identical with the vitamin E deficiency seen in laboratory animals (Willman et al., '34; Mackenzie et al., '41) and the disease can be prevented or cured in the early stages by feeding alpha-tocopherol. Mason and Bryan ('38, '40) found that vitamin E supplementation increased the stores of vitamin E in the young and the milk of rats. Other investigators (Evans and Burr, '38; Olcott, '38; Pappenheimer, '42) have shown that the young of vitamin E-deficient rats and mice developed symptoms of vitamin E deficiency during the first few weeks of life, while young from normally-fed females showed no such symptoms. More recently, Parrish et al. ('47) have shown that supplementing the ration of dairy cows with 500 to 1000 mg of tocopherols daily during the last 4 weeks of pregnancy increased the tocopherol content of the colostrum from 107 to 150 μ g per gram of fat, and that feeding 10 gm of tocopherols daily increased the colostrum content to 487 μ g per gram of fat.

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These studies suggested that the incidence and severity of the dystrophy mentioned above might be influenced by the placental and mammary transfer of alpha-tocopherol. Because of the lack of published data on this subject, it seemed worthwhile to make a study using farm animals.

EXPERIMENTAL PROCEDURE

Forty ewes, 16 goats and 25 sows were used in this study. The ewes and goats were fed a ration consisting of second-cutting mixed alfalfa and clover hay and red kidney beans. Half of them received, in addition, a daily supplement of 80 mg of mixed tocopherols² per 100 lb. of body weight. The pigs were fed a ration of 760 parts of ground yellow corn, 100 of soybean oil meal, 100 of ground alfalfa hay, 30 of meat scrap, and 10 of minerals. Half of the sows received the daily tocopherol supplement of 80 mg per 100 lb. of body weight.

At the time of parturition the tocopherol supplement was removed from the diets. Samples of liver and blood were obtained from the newborn animals within a few hours after birth. Care was taken to use only the young which were removed from their dams before they had sucked. Colostrum samples were taken before the mothers had been nursed. Milk was collected from the same females again 4 days after parturition. The method of Quaife and Harris ('44) that employs the microhydrogenation technique of Quaife and Biehler ('45), was used to determine the tocopherol content of blood plasma. The method of Quaife ('47) was used to determine the tocopherol content of colostrum and milk. The method of Hines and Mattill ('43), modified as indicated below, was used to determine the tocopherol content of livers. Instead of using the florisol XS earth adsorption column for removing interfering compounds after the H_2SO_4 and KOH treatments, the sample was taken up in a 50:50 mixture of cyclohexane and alcohol, and hydrogenated. Tocopherols were then measured in the same manner as for plasma and milk except that the

² The vitamin E supplement, "Myvadry," was generously supplied by Distillation Products, Inc., Rochester, New York.

absorption reading was taken after a one-minute reaction time. Essentially stable readings were obtained between one and 10 minutes if all light was excluded, indicating, according to Baxter ('47), that probably the non-tocopherol reducing substances had been removed.

RESULTS AND DISCUSSION

The tocopherol contents of the livers and blood plasma of newborn lambs, kids and pigs are shown in table 1.

While parturition tocopherol supplementation at the levels used increased the average tocopherol content of the livers of the newborn animals, the increases were not statistically sig-

TABLE 1

The influence of diet on the tocopherol content of liver and plasma of newborn animals

SPECIES	TOCOPHEROL CONTENT			
	Liver		Plasma	
	Controls	Supplemented	Controls	Supplemented
	$\mu\text{g/gm}$		$\mu\text{g/100 ml}$	
Lambs	(6) ¹ 25.3 \pm 2.7	(6) 30.0 \pm 4.3	(6) 20 \pm 10	(6) 94 \pm 62
Kids	(5) 10.4 \pm 1.5	(6) 13.1 \pm 2.0	(5) 16 \pm 8	(5) 65 \pm 40
Pigs	(8) 24.7 \pm 3.6	(10) 26.4 \pm 4.7	(6) 120 \pm 28	(7) 101 \pm 33

¹ Figures in parenthesis are the numbers of samples analyzed.

nificant (5% level of probability). This may indicate either that liver storage of tocopherol in the newborn cannot be significantly increased by parturition tocopherol supplementation or that greater amounts are required to cause significant increases. It is also possible that the liver may not be the site of richest tocopherol storage, since Mason ('42) and Abderhalden ('45a) have shown that vitamin E is widely distributed throughout the animal body. It is interesting that Abderhalden reported the liver of the newborn infant to contain 4.60 μg of tocopherols per gram of tissue; two livers of newborn calves analyzed by the authors showed an average tocopherol content of 13.8 μg per gram of fresh liver tissue. No other values

for the tocopherol content of the livers of newborn mammals were found in the literature.

The tocopherol content of the blood plasma of newborn lambs and kids was significantly increased (1% level of probability) by prepartum vitamin E supplementation, but no increase occurred in the pigs (table 1). This may be explained, in part, on the basis that the ration fed to the ewes and goats was deficient in tocopherol activity (Willman et al., '45, '46), whereas the pig's ration was adequate; thus, the former animals might be expected to show a greater response to supplemental tocopherols than the sows. Abderhalden ('45b) reported that the blood plasma of the newborn infant contained

TABLE 2

The influence of diet on the tocopherol content of colostrum and milk of sheep, goats and pigs

SPECIES	TOCOPHEROL CONTENT			
	Colostrum		Milk	
	Controls	Supplemented	Controls	Supplemented
	<i>μg/gm fat</i>			
Sheep	(19) ¹ 47 ± 20	(14) 78 ± 25	(19) 15 ± 6	(15) 30 ± 7
Goats	(5) 59 ± 18	(8) 154 ± 10	(5) 14 ± 5	(8) 31 ± 13
Pigs	(10) 186 ± 61	(10) 399 ± 175		

¹ Figures in parentheses are the numbers of samples analyzed.

an average of 96 μg of tocopherols per 100 ml of plasma. Straumfjord and Quaife ('46) on the other hand reported a plasma content of 340 μg of tocopherols per 100 ml for the newborn infant.

The tocopherol content of the colostrum and milk of the ewe, goat and sow is shown in table 2.

Prepartum tocopherol supplementation significantly increased the tocopherol content of the colostrum of all three species. In the case of the sows and goats, the increase was highly significant (1% level of probability), but the varia-

bility among animals on the same ration was very large. Parrish et al. ('47) made a similar observation from their data on the tocopherol content of cow's colostrum and milk. Sow's colostrum contained about three times as much tocopherol as that of the goats and ewes, and somewhat more than that reported by Parrish et al. ('47) for cows fed a normal ration. Colostrum from ewes fed a more standard ration of mixed hay, corn silage and cereal grains contained an average of 106 μ g of tocopherols per gram of fat. Quaife ('47) reported that human colostrum collected during the first week after parturition contained 0.13 to 3.6 mg of tocopherol per 100 ml. Abderhalden ('44) found that human colostrum taken two days after childbirth contained an average of 3.3 mg per 100 gm, and that at 4 to 10 days postpartum the milk contained 0.94 mg per 100 gm.

Milk of the ewe and goat contained approximately one-fourth as much tocopherol as the colostrum of these animals. This is in agreement with the findings of Parrish et al. ('47) for dairy cows. The milk of the ewes and goats that received tocopherol supplements prepartum contained approximately twice as much tocopherol as that of non-supplemented ewes and goats.

SUMMARY

The placental transfer and colostrum storage of total tocopherols was investigated in sheep, goats and swine. Supplementing the prepartum ration with 80 mg of mixed tocopherols per 100 lb. body weight slightly increased the liver storage of tocopherols in the newborn animal, but the increase was not statistically significant. A highly significant increase in the tocopherol content of the blood plasma of the lambs and kids resulted from the prepartum supplementation, but no increase was observed in swine. Prepartum supplementation caused a two-fold increase in the tocopherol content of the colostrum in all species. Colostrum contained three to 4 times as much tocopherol as the milk obtained from the same animals 4 days later.

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INFLUENCE OF THE CONCENTRATION OF MIXTURES OF VARIOUS COMPONENTS OF THE VITAMIN B COMPLEX ON BIOLOGICAL VALUE OF CASEIN AND ON ECONOMY OF FOOD UTILIZATION ¹

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There is ample evidence in the literature that the various components of the vitamin B complex play significant roles in nitrogen metabolism. Urinary studies of animals subsisting on diets lacking members of the complex show changes in the excretion of ammonia, urea, creatine, uric acid and allantoin, and examination of the blood reveals alterations in concentrations of non-protein nitrogen and other factors (Sure and Dichek, '41; Sure and Ford, '42). The finding by Gunsalus and Bellamy ('44) and Bellamy et al. ('45) that pyridoxine functions as a co-factor in amino acid decarboxylases suggests a role for this B vitamin in protein metabolism. The relationship between tryptophane and niacin studied by Krehl and colleagues ('46a, '46b), Schweigert et al. ('47, '48) and Salmon ('47) can also be cited as pertinent in this connection.

The purpose of the present study was to investigate the influence of the concentration of synthetic mixtures of the

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vitamin B complex on protein efficiency and on economy of total food utilization. The biological values of purified casein² fed at different planes of intake and supplemented with graduated increasing concentrations of mixtures of various components of the vitamin B complex were determined by the nitrogen balance methods of Mitchell ('24, '44).

EXPERIMENTAL

The percentage composition of the rations was as follows: Casein, 8.2 to 20; cellu flour, 2; Sure's salts no. 1 (Sure, '41), 4; lard, 10; cod liver oil, 1.5; wheat germ oil, 0.5; and the balance, cerelose. Synthetic mixtures of pure crystalline com-

TABLE 1
Composition of vitamin B complex mixtures

	1	2	3	4	5	6	7
Thiamine	3 μ g	5 μ g	10 μ g	15 μ g	25 μ g	50 μ g	100 μ g
Riboflavin	3 μ g	5 μ g	10 μ g	15 μ g	25 μ g	50 μ g	100 μ g
Pyridoxine	3 μ g	5 μ g	10 μ g	15 μ g	25 μ g	50 μ g	100 μ g
Niacin	3 μ g	5 μ g	10 μ g	15 μ g	25 μ g	50 μ g	100 μ g
Calcium pantothenate	25 μ g	50 μ g	75 μ g	100 μ g	150 μ g	300 μ g	600 μ g
<i>p</i> -aminobenzoic acid	250 μ g	500 μ g	1 mg	2 mg	3 mg	6 mg	12 mg
Inositol	75 μ g	150 μ g	300 μ g	600 μ g	1 mg	3 mg	3 mg
Choline chloride	3 mg	6 mg	6 mg	6 mg	6 mg	9 mg	12 mg

ponents of the vitamin B complex which have proved to be essential for growth of the rat were used. The composition of these mixtures is given in table 1. Six rats were used in each group of experiments. However, the findings on animals which showed the greatest variations from the mean were eliminated, so that in table 2 results are shown on 5 animals and in table 3 on 4 animals. In table 4 results are submitted on 6 animals.

² Smaco.

Urinary and fecal balances were determined over periods of 7 days on an egg standardizing ration and then for 7 days on the experimental rations containing 8.2, 10, and 20% casein; these rations contained 7.1, 8.7, and 17.3% protein, respectively. The 8.2 and 10% casein rations were supplemented with 25 mg cystine per animal per day. Accurate records of food consumption were kept, so it was possible to calculate (1) the protein efficiency ratios, expressed as gain in weight per gram of protein intake; (2) the biological values, expressed as the per cent of absorbed nitrogen retained by the rat; and (3) the gain in body weight per 100 gm of food intake, which expressed the requirements for maintenance and growth and was the yardstick we used for evaluating economy of food utilization.

Before beginning the nitrogen balance studies, the rats were depleted of the vitamin B complex for 10 to 15 days until either maintenance was reached or there were slight losses in body weight. In starting the metabolism experiments the animals were 25 to 28 days of age and weighed about 50 gm.

In the construction of the vitamin B complex synthetic mixtures we used for the daily doses of thiamine, riboflavin, pyridoxine, and niacin, 3 to 100 μ g; for calcium pantothenate, 25 to 600 μ g; for *p*-aminobenzoic acid, 250 μ g to 12 mg; for choline chloride, 3 to 12 mg; and for inositol, 75 μ g to 3 mg (table 1). These mixtures (VBC)³ are hereinafter referred to either as the numbers 1 to 7 given them in table 1 or as mixtures having the 3, 5, 10, and 15 μ g doses, since the same daily doses were used for 4 components of the vitamin B complex.

RESULTS

Data from these experiments are submitted in tables 2, 3, and 4. It will be noted from table 2 that, on a 20% casein intake, increasing various components of the vitamin B complex produced no notable changes in nitrogen retention during

³ VBC = vitamin B complex.

TABLE 2

Influence of concentration of the vitamin B complex (VBC) on biological values of protein and on economy of food utilization (Average results for 5 animals in each group, 3 ♂'s and 2 ♀'s)

PROTEIN	VBC MIXTURES ¹	DAILY DOSE OF VIT. B COMPLEX FLAVIN AND NICOTIN	GAIN IN BODY WEIGHT	TOTAL FOOD INTAKE	INCREASE IN FOOD INTAKE	GAIN IN BODY WEIGHT PER 100 GM FOOD INTAKE	INCREASE IN FOOD UTILIZATION	PROTEIN EFFICIENCY RATIO ²	BIOLOGICAL VALUE ³
		μg	gm	gm	%	%	%		
20%	1	3	6.6	32.3	...	20.4	.	1.19 ± 0.15^4	61.6 ± 4.66^5
Casein ⁴	2	5	10.0	38.2	18.3	26.2	28.4	1.64 ± 0.18	62.6 ± 2.52
(17.3% protein)	3	10	21.2	46.8	44.9	45.3	122.1	2.61 ± 0.07	63.2 ± 2.00
	4	15	16.6	42.9	32.8	38.7	89.7	2.44 ± 0.06	59.0 ± 6.45
10%	1	3	3.8	31.5	.	12.1	..	1.63 ± 0.23	71.8 ± 3.55
Casein ⁴	2	5	9.2	37.1	17.8	24.8	105.0	2.87 ± 0.17	79.0 ± 2.19
(8.7% protein) +	3	10	18.2	47.7	51.4	38.2	215.7	4.40 ± 0.07	77.5 ± 3.35
25 mg cystine/ rat/day	4	15	22.8	53.6	71.4	42.5	251.2	4.79 ± 0.31	77.7 ± 3.87

¹ See table 1.² Gain in body weight per gram of protein intake.³ Per cent of absorbed nitrogen retained in the body.⁴ Smaco.⁵ Standard deviation of the means.⁶ Standard deviation (Sherman, '41).

the 7-day metabolism period. However, a change from VBC mixture 1 to 3 resulted in an increase of about 50% in food intake and of over three times the gain in body weight. The increase of 119% in the so-called "protein efficiency ratio" parallels the increase of 122% in total food utilization. On a 10% plane of casein intake, increasing the daily doses of thiamine, riboflavin, pyridoxine, and niacin from 3 to 15 μ g, with the same increased doses of the rest of the components of the vitamin B complex mixtures, also produced no significant changes in nitrogen retention during the one-week balance study. On the other hand, there was a 70% increase in food intake and a 6-fold gain in body weight, which establish conclusively that the enhanced growth following the increases in the concentration of the vitamin B complex was entirely out of proportion to the rise in food consumption and the marked gains in food utilization should be ascribed to the greater intake of the B vitamins. The increase from the 3 to 15 μ g doses produced a rise of from 1.63 to 4.79, or 193%, in the so-called "protein efficiency ratio," and an increase of 251% in total food utilization.

On an 8.2% level of casein intake, introducing 7.1% protein in the ration, the influence on growth and food utilization was most marked. The increase from the 3 to the 5 μ g daily doses (table 3), produced a marked increase in nitrogen retention. This is undoubtedly due to the increased caloric intake associated with the increased concentration of the vitamin B complex mixture.

The high figure for the standard deviation of the biological value on VBC mixture 1 during the first metabolism period (table 3) is due to the great variations in food intake and to variations in the small increments of growth on this low mixture of the vitamin B complex.

Following a standardizing period, the animals which were receiving VBC mixtures 1, 2, 3, and 4 were given, respectively, VBC mixtures 4, 5, 6, and 7, thus increasing the daily doses of thiamine, riboflavin, pyridoxine, and niacin from 3, 5, 10, and 15, to 15, 25, 50, and 100 μ g. This shift to higher doses of

TABLE 3

Influence of concentration of the vitamin B complex (VBC) on biological values of protein and on economy of food utilization
(Average results for 4 animals in each group, 2 ♀'s and 2 ♂'s)

PROTEIN	VBC MIXTURES ¹	DAILY DOSES OF THIA-MINE, RIBO-FLAVIN AND NIACIN	GAIN IN BODY WEIGHT	TOTAL FOOD INTAKE	INCREASE IN FOOD INTAKE	GAIN IN BODY WEIGHT PER 100 GM FOOD INTAKE	INCREASE IN FOOD UTILIZATION	PROTEIN EFFICIENCY RATIO ²	BIOLOGICAL VALUE ³
		μg	gm	gm	%	gm	%		
8.2%	1	3	0.75	26.8	...	21.8	...	0.68 ± .17 ³	46.3 ± 8.4 ⁴
	2	5	6.5	37.8	41.1	17.2	514.3	2.37 ± .62	81.6 ± 3.5
Casein ⁴	3	10	17.2	48.1	79.5	35.8	1178.6	4.94 ± .63	85.2 ± 2.7
(7.1% protein)	4	15	22.2	57.4	114.2	30.7	1282.2	5.51 ± .44	85.0 ± 1.3
+ 25 mg cystine/rat/day	4	15	17.0	61.6	Second metabolism periods ¹				
	5	25	23.0	73.8	19.8	27.6	...	3.88 ± .36	72.7 ± 3.66
	6	50	27.0	80.8	31.2	31.2	13.0	4.42 ± .39	69.5 ± 4.67
	7	100	23.2	76.5	24.2	33.4	21.0	4.73 ± .22	70.2 ± 2.64
						30.3	9.8	4.28 ± .35	64.3 ± 3.56

¹ See table 1.² Gain in body weight per gram of protein intake.³ Per cent of absorbed nitrogen retained in the body.⁴ Smaco.⁵ Standard deviation of the means.⁶ Standard deviation (Sherman, '41).

⁷ Following a standardizing period, the animals which received vitamin B mixtures 1, 2, 3, 4, were given, respectively, vitamin B mixtures 4, 5, 6, and 7 during the second one-week metabolism period.

the vitamin B complex, even on the lower level of protein intake (7.1%) resulted in no change in efficiency of nitrogen retention, but this change in vitamin concentration was accompanied by a marked increase in body weight and in food consumption. Optimum results were obtained on VBC mixture no. 6. A further increase in the vitamin B complex, from this mixture to no. 7, produced a depression in growth and in efficiency of protein utilization. These results may have practical applications in the war-torn countries abroad where pronounced food shortages still exist. On the basis of data presented in table 3 it appears that on a low protein level (7.1%) some growth was possible but only when the various components of the vitamin B complex were raised to high concentrations. On the same low protein intake and with a low concentration of the B vitamins, only maintenance was obtained. These observations were made during a one-week experimental period. During longer periods on the low vitamin B complex and low protein diet, these animals, undoubtedly, would have collapsed. At least, experiences in this laboratory make this seem probable.

A study was also carried out on the influence of concentration of the vitamin B complex on the chemical composition of body gains. Twelve male and 12 female rats were used for this investigation. They were a month old and weighed 50 to 59 gm each when started on experiment. They were depleted of the vitamin B complex for 12 to 15 days, were placed on a preliminary vitamin period for several days to get accustomed to the new dietary regimen, and then were kept for 21 days on a 10% casein diet with graduated increasing doses of VBC mixtures 1, 2, 3, and 4. In addition, 12 male and 12 female rats of the same initial age and weight were taken one week after weaning from stock diets and analyzed for moisture, protein, fat, and ash. From the chemical compositions and body weights at the beginning and at the end of the experimental periods the chemical composition of body gains was computed. The results are submitted in table 4. It will be noted that during this three-week period a change from

VBC mixture 1 to 2, increasing from the 3 to the 5 μ g doses, resulted in increased protein synthesis, with no further increased protein synthesis following further increases of vitamin B complex concentrations. However, synthesis of fat resulted from the further change from VBC mixture 2 to 3, when the daily doses were increased from 5 to 10 μ g. The marked increases in growth following increase in certain concentrations of the vitamin B complex must then be due to fat synthesis from the carbohydrate (cerelose) in the rations. The only notable change resulting from raising the daily doses from 10 to 15 μ g was in the ash content; we have no explanation for the reduction in ash when the VBC mixture was changed from 2 to 3, increasing the daily doses from 5 to 10 μ g.

The fact that we observed in this investigation frequent parallelisms between per cent increase in food utilization and per cent increase in protein efficiency ratios and, in most cases, no parallelism between biological values (as determined from nitrogen balance studies) and protein efficiency ratios, would indicate that the latter do not always express solely the gains in weight per gram of protein intake. The so-called "protein efficiency ratios" must also include gains in body weight pro-

TABLE 4

Influence of concentration of the vitamin B complex (VBC) on chemical composition of body gains¹ (Average results for 6 animals in each group, 3 ♀'s and 3 ♂'s)

VBC MIXTURES ¹	DAILY DOSES OF THIAMINE, RIBOFLAVIN AND NIACIN	GAIN IN BODY WEIGHT ²	INCREASE IN BODY WEIGHT	GAIN IN BODY WEIGHT PER 100 GM FOOD INTAKE	INCREASE IN FOOD UTILIZATION	PROTEIN EFFICIENCY RATIO	CHEMICAL COMPOSITION OF BODY GAINS		
							Ash	Fat	Protein
	μ g	gm	%	gm	%		gm	gm	gm
1	3	22.0	...	16.7	...	1.95 \pm .16 ³	+ 1.69	+ 0.93	+ 5.06
2	5	29.0	31.8	18.5	10.8	2.15 \pm .10	+ 2.15	+ 3.70	+ 8.65
3	10	41.0	86.4	22.3	33.5	2.58 \pm .10	+ 1.89	+ 6.26	+ 8.75
4	15	41.7	80.6	22.0	31.7	2.54 \pm .07	+ 2.47	+ 5.81	+ 9.09

¹ See table 1.

² Experimental period of 21 days. The animals received a 10% casein diet, supplemented with 25 mg cystine per animal per day.

³ Standard deviation of the means (Sherman, '41).

duced by caloric intakes from the ration. Our observations are in agreement with those of Barnes and Bosshardt ('46) who state that "... the influence of caloric intake upon the growth utilization of protein may be greater than has been suspected, heretofore." Bosshardt and associates ('46b) in their recent protein studies with mice fed ad libitum found that "... with each protein source the level of intake exhibiting maximal protein utilization corresponded with a maximal caloric intake per unit of body surface area, and at a given level of protein intake changes in caloric consumption often resulted in the apparent utilization of protein."

A study was also made of the influence of aqueous butyl alcohol extracts of Wilson's 1:20 liver concentrate powder on the utilization of casein for growth. The casein was fed at a 10% plane of intake and the extracts of the liver concentrate were prepared by the procedure of Conger and Elvehjem ('41). A total of 48 rats was used in this investigation. The extracts were fed at 0.5 and 0.2% of the rations. It was anticipated that such extracts might furnish unknown components of the vitamin B complex. Following 11 weeks' growth, the results were entirely negative. Such extracts also resulted in no increases in nitrogen retention, as evidenced by balance studies. Our observations on rats are, therefore, not in accord with those of Bosshardt and co-workers on mice ('46a).

SUMMARY

Increasing the concentrations of thiamine, riboflavin, pyridoxine, niacin, pantothenic acid, choline, *p*-aminobenzoic acid, and inositol used in synthetic mixtures as sources of the vitamin B complex resulted in marked increases in growth and pronounced increases in food utilization. However, increases in nitrogen retention and protein synthesis occurred only within a narrow range of the low concentration of the vitamin B complex. The marked increases in growth are due largely to fat synthesis from the carbohydrate (cerelose) in the rations.

On a low 7.1% protein level some growth was possible, but only when the various components of the vitamin B complex were raised to high concentrations. On the same low protein intake and on a low concentration of the B vitamins, only maintenance was obtained. These observations were made during a one-week experimental period.

The results of feeding aqueous butyl alcohol extracts of 1:20 Wilson's concentrate powder as a source of unknown components of the vitamin B complex were negative as far as utilization of casein for growth is concerned.

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ANEMIA AND EDEMA OF CHRONIC CHOLINE DEFICIENCY IN THE RAT¹

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FOUR FIGURES

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For some years this laboratory has conducted investigations to determine the effects of choline deficiency in the rat. During these studies it was observed that neoplasms develop in the livers and other tissues in a significant number of animals subjected to such a deficiency for prolonged periods (Copeland and Salmon, '46; Engel, Copeland and Salmon, '47). It seemed desirable to determine if some physiological abnormality might appear in such animals during the development of the chronic choline deficiency syndrome. A lowered hemoglobin has been reported in dogs fed choline-deficient diets (Fouts, '43; McKibbin et al., '44). Attention was therefore directed to the blood; hemoglobin determinations were made on rats in an experiment in which a variety of choline-low and/or protein-low diets were under investigation.

The production of anemia as well as nutritional edema as a result of choline deficiency will be discussed in the following pages.

EXPERIMENTAL

Rats of the Alabama Experiment Station (AES) strain were used. The animals were fed the experimental diets from

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the age of 23 days, except in one case in which older animals were used. Care was exercised to maintain litter and sex controls in studying treatment effects. The animals were housed individually in screened-bottom cages, and were supplied with fresh food daily.

The percentage composition of the diets used in these studies is given in table 1. The diets were stored in a refrigerator and were replenished about once every 10 days.

TABLE 1
Percentage composition of the basal diets¹

INGREDIENT	DIET NO.						
	46E	57	C-1	46	59	58	60
Extracted casein ²	4.5	6.5	9.0	9.0	9.0	0.0	6.0
Extracted peanut meal ³	0.0	0.0	0.0	0.0	0.0	30.0	30.0
Degerminated corn grits ⁴	40.0	40.0	20.0	40.0	30.0	30.0	0.0
Salts ⁵	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Sucrose	36.2	34.2	51.7	31.7	36.7	15.9	39.9
Lard	14.0	14.0	14.0	14.0	19.0	19.0	19.0
Cod liver oil	1.0	1.0	1.0	1.0	1.0	1.0	1.0
L-cystine	0.3	0.3	0.3	0.3	0.3	0.1	0.1

¹ Each kilogram of basal diet was fortified with the following: 2 mg each of thiamine and pyridoxine, 4 mg of riboflavin, 10 mg of calcium pantothenate, 20 mg of niacin, 200 mg of *D*-inositol and 50 mg of alpha-tocopherol. Control animals received a further supplement of 2000 mg of choline chloride per kilogram of diet. The author is indebted to Merck and Company, Rahway, New Jersey, for a supply of these vitamins.

² Commercial casein was purified by percolation with tap water for 6 days with overnight acidifications (0.2% acetic acid) and finally washed with 95% alcohol and dried. The purified product contained 87% protein ($N \times 6.38$).

³ Commercial peanut meal was percolated exhaustively with water solution containing 60% ethanol by volume and finally extracted with absolute methanol and dried. The extracted material contained 44% crude protein ($N \times 6.25$).

⁴ A non-enriched commercial product containing 8.8% crude protein ($N \times 6.25$).

⁵ J. Nutrition, 33: 155-168, 1947.

Effect of choline or methionine on hemoglobin

Hemoglobin determinations were made at least once monthly. The samples were obtained by bleeding from the tail. The acid hematin method was used, and the color den-

sity was measured with filter 420 in an Evelyn photoelectric colorimeter. The instrument was calibrated with graded concentrations of pure hematin prepared from beef blood according to the method of Clifcorn and associates ('35).

The results of these analyses are summarized in table 2. A lowered hemoglobin with a range of 6.25 to 11.95 gm per 100 ml blood was consistently observed in animals maintained on the choline-deficient diets for three months or longer.

TABLE 2

Final hemoglobin concentration and body weight of rats fed diets varying in protein and fat with and without supplement of choline or methionine (6-month experimental period)

NO. OF ANIMALS PER TREATMENT	SEX	DIET NO.	TREATMENT				DIET- ARY PRO- TEIN	DIET- ARY FAT
			Choline added		No choline added			
			Body wt.	Hemo- globin	Body wt.	Hemo- globin		
			gm	gm/100 ml blood	gm	gm/100 ml blood	%	%
2	M	46E ¹	546	13.60	339	6.25	7.5	15
3	F	46E ²	339	14.20	335	6.85	7.5	15
6	M	57	388	13.45	353	8.75	9.2	15
2	F	57	271	13.85	317	10.05	9.2	15
3	M	C-1	438	14.40	320	8.55	9.6	15
2	F	C-1	265	14.25	283	11.80	9.6	15
2	M	C-1 ³	445	14.25	444	14.90	9.9	15
2	F	C-1 ³	277	13.85	260	14.55	9.9	15
2	M	46	472	13.00	403	10.10	11.4	15
2	F	46	287	13.85	320	11.95	11.4	15
2	M	46 ³	515	12.20	451	13.75	11.7	15
2	F	46 ³	303	14.55	339	14.70	11.7	15
2	M	59	459	14.30	284	7.85	10.5	20
2	M	58	404	13.80	202	9.85	15.8	20
2	F	58	223	14.65	178	10.05	15.8	20
2	M	60	431	14.30	309	10.30	19.2	20

¹ These animals were placed on diet 46E after they had received diet 46 for 6 months after weaning.

² These animals were placed on diet 46E after 9-12 months on a normal stock diet.

³ These animals received a supplement of 3 gm of DL-methionine per kilogram of diet.

Male animals generally became slightly more anemic and made poorer weight gains on the deficient diets than females. The severity of the anemia appeared to be correlated with the protein content of the diet, the lowest hemoglobin levels occurring in the animals receiving the diets most deficient in protein. The important finding, however, is that the anemia was prevented by choline or methionine even at the lowest protein levels.

The anemia developed within the first three months after the animals were placed on the diets unsupplemented with choline or methionine. The hemoglobin concentration remained essentially unchanged at the lowered levels between

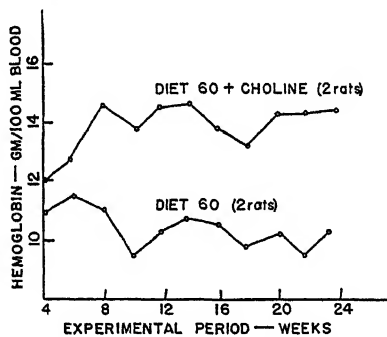


Fig. 1 Average hemoglobin concentration at 2-week intervals in rats fed diets with and without added choline.

the third and 6th months. In one study, hemoglobin determinations were made at two-week intervals between the 4th and the 24th week in order to follow more closely the development of the anemia. The results are presented in figure 1. Since rats of this strain do not survive if dietary choline is not supplied during the period of rapid growth, it was our practice to supply animals on the choline-deficient diets with enough choline to prevent fatal kidney damage during the first month of the experiment. In the case of the choline-deficient animals represented in figure 1 this requirement was met by supplementing the diet with 650 mg of choline chloride per kilogram. As can be seen in this figure, the

choline-deficient animals became anemic rather rapidly between the 6th and 10th weeks of the experiment. The lowered hemoglobin levels remained more or less unchanged during the subsequent 14-week period.

*Ineffectiveness of folic acid in curing the
choline-deficiency anemia*

Since the diets did not contain a supplementary source of folic acid, it seemed desirable to determine the effectiveness of this factor in the anemia that results from lack of choline. For this purpose animals that had developed the anemic condition while subsisting on choline-deficient diet 57 for 4 months were given a supplement of 2 mg of the sodium salt of pteroyl-diglutamic acid² per kilogram of diet. There was no improvement during the subsequent 4-month period. Hemoglobin levels remained unchanged and were comparable to those observed in litter mates not receiving the supplement.

Edema in chronic choline deficiency

The animals receiving the choline-deficient diet lowest in protein, Diet 46E, became more anemic than did animals on diets less restricted in protein. It was observed that a marked accumulation of fluid occurred in the abdominal and thoracic cavities as well as in the subcutaneous tissues of some of the animals receiving Diet 46E without choline supplement. To date this marked edema has been observed in 7 of 12 animals receiving this diet for 4 months or longer. One case of severe edema has been observed in an animal receiving choline-deficient Diet 57, a diet similar to Diet 46E except that it contained 6.5% casein instead of 4.5%. This edematous condition has not been observed in animals receiving the same diets supplemented with choline.

The severity of this edema is illustrated in the photographs in plate 1. The edema was consistently accompanied by severe

² The Laboratory is indebted to Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Company, for a supply of this compound.

hepatic cirrhosis evidenced grossly by an irregular, nodular liver surface.

DISCUSSION

It is possible that the function of choline in preventing anemia in these experiments is that of sparing an amino acid essential for the synthesis of hemoglobin protein; namely, methionine. The effectiveness of DL-methionine in preventing anemia in the present study supports this hypothesis. Robscheit-Robbins and associates ('47) have demonstrated an increased output of blood proteins in protein-depleted dogs by the addition of methionine to an otherwise complete amino acid mixture. Albanese and co-workers ('46) observed a reduction in hemoglobin in rats fed diets nearly devoid of methionine. Li and Freeman ('47) fed rats a diet containing 9% casein as the source of protein and observed a marked growth stimulation and a slight rise in hemoglobin when the diet was supplemented with methionine. These investigators relied upon brewers' yeast at a level of 0.5 gm per rat per day as the source of choline. The adequacy of choline intake could be questioned in such a diet unsupplemented with methionine, since that amount of yeast would supply only about 2.5 mg of choline per rat per day (Engel, '42). The effectiveness of choline in preventing the severe anemia which occurred on diets containing as low as 4.5 or 6.5% casein in the present study would suggest either a striking protein-sparing property of this nutrient or some still unknown relationship between it and hematopoiesis.

The possibility must be considered that the severely damaged liver resulting from chronic choline deficiency may in some way interfere with normal hemoglobin or red blood cell formation. Moosnick and associates ('45) have made the interesting observation that choline may have therapeutic value in certain human anemias. They treated a pernicious anemia patient refractory to purified liver extract with intravenous choline and noted a definite response. Prior to treatment the patient showed fatty metamorphosis of the liver. Davis and Brown ('47) have recently reported on the use of

choline in megaloblastic anemias. They observed a significant response to choline in two cases resembling Addisonian pernicious anemia which were refractory to parenteral liver extracts.

It is possible that there may be a relationship between the lowered hemoglobin and the development of neoplasms in rats subjected to a chronic choline deficiency. Other investigators, in studying pre-cancerous changes in animals, have noted a correlation between tumor induction and hemoglobin level. Strong and Frances ('40) observed a gradual drop in hemoglobin in a highly tumor-susceptible strain of mice during the pre-cancerous stages, an abnormality which did not occur in mice of a strain relatively resistant to mammary tumor development. Taylor and Pollack ('42) likewise reported lowered hemoglobin during the pre-cancerous stage in mice injected with methylcholanthrene and also in rats fed diets containing *p*-dimethylaminoazobenzene.

The occurrence of prominent symptoms of nutritional edema with severe ascites in rats fed low-protein diets unsupplemented with choline raises a question as to the possible importance of choline in nutritional edema in the human. This disease is usually associated with an inadequate protein intake. Under such conditions the choline intake likewise would probably be inadequate. It is significant that in the present study the edema was consistently accompanied by severe liver damage, a condition which is not uncommon in human nutritional edema. The method reported here for the experimental production of nutritional edema should be valuable for carefully examining the therapeutic value of choline or related nutrients in the control of this disease.

SUMMARY

Prolonged feeding of diets deficient in choline produced an anemia in rats. The hemoglobin levels in the deficient animals ranged from 6.25 to 11.95 gm per 100ml of blood. The anemia was prevented by dietary supplements of choline

chloride or DL-methionine. The sodium salt pteroyl-di-glutamic acid was ineffective as a curative agent.

The prolonged feeding of diets low in protein and choline resulted in symptoms of severe nutritional edema in 7 of 12 rats. This condition was not observed in control animals receiving the same diets supplemented with choline.

ACKNOWLEDGMENT

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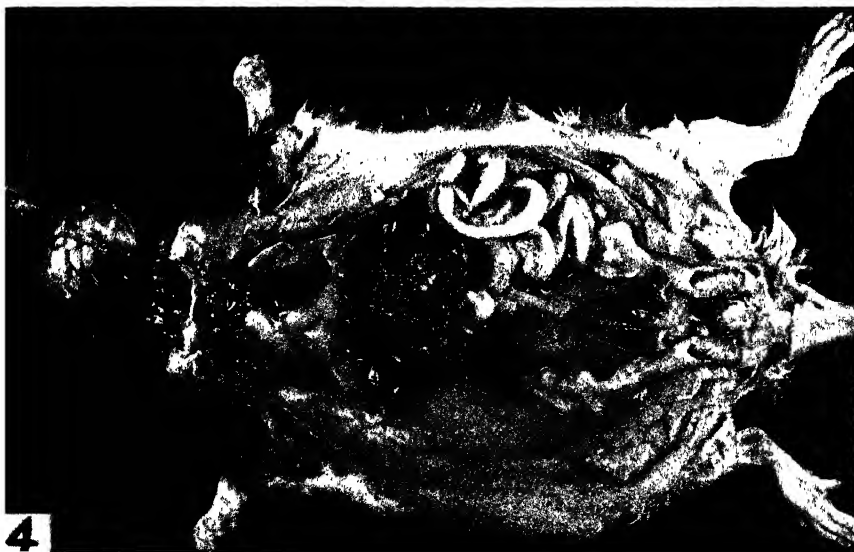
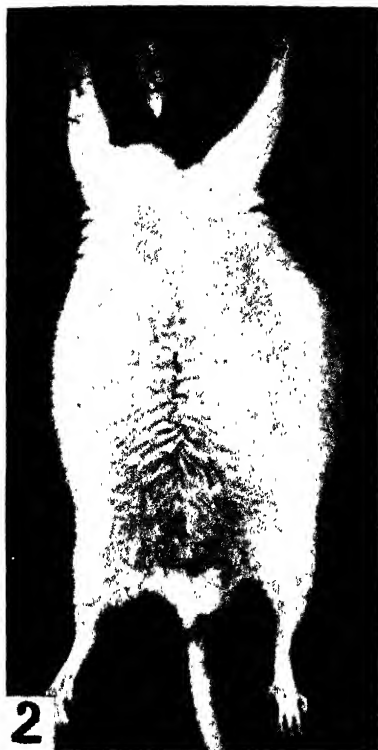
PLATE 1

EXPLANATION OF FIGURES

2 Gross nutritional edema with marked ascites in a rat which received Diet 57 without choline for 11 months.

3 Same rat with a superficial dissection to illustrate fluid accumulation throughout the subcutaneous tissues and extensive ascites as evidenced by a severely distended abdomen.

4 Same rat with thoracic and abdominal cavities exposed. At autopsy 169 gm of a clear, slightly yellow fluid was drained from the abdominal cavity of this animal which weighed 380 gm at death. Note the cirrhotic condition of the liver and the severely atrophied testes. There is a complete absence of visceral fat stores in this condition.



GROWTH EFFICIENCY OF ESSENTIAL AMINO ACIDS ALONE AND IN COMBINATION WITH CASEIN^{1,2}

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ONE FIGURE

(Received for publication June 28, 1948)

The well-known report of Rose ('37) concerning the indispensability of certain amino acids for growth of the white rat, and his estimation of minimum requirements, have given rise to a number of studies in this field, some of which will be referred to in the discussion of the results of the present experiment.

The objectives of the present study were: (1) to test again the growth-promoting value of the 10 essential amino acids when fed as the sole source of nitrogen, at the levels suggested by Rose; (2) to determine the effectiveness of the essential amino acids when fed in part as casein and in part in the crystalline form; and (3) to determine the effects of feeding the essential amino acids, as the sole source of nitrogen, at levels higher than the suggested minimum.

EXPERIMENTAL

Diets of the purified type were used and the ingredients common to all diets were the following: corn oil, 9%; cod

¹ Journal Series paper of the Department of Agricultural Biochemistry, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick.

² Presented before the Division of Biological Chemistry, American Chemical Society, New York, N. Y., September, 1947.

liver oil, 1%; sodium chloride, 1%; and salt mixture, U. S. P. No. 1, 4%. Crystalline amino acids were ground with cane sugar to make 10% or 20% of the diet, along with the quantity of sodium bicarbonate necessary to neutralize the hydrochloric acid of arginine, histidine and lysine hydrochlorides. Casein was used in the amounts indicated in table 1, and the balance of each diet consisted of corn starch. Water soluble vitamins and other organic factors were added in the following quantities per 100 gm of diet: thiamine, 0.5 mg; riboflavin, 1.0 mg; pyridoxine, 0.5 mg; calcium pantothenate, 2.5 mg; nicotinic acid, 2.0 mg; inositol, 0.25 mg; *p*-aminobenzoic acid, 5.0 mg; choline, 20 mg; biotin, 2 μ g; and folic acid, 0.1 mg.

Each test group consisted of 2 male and 2 female white rats, 21-24 days of age and weighing between 55 and 65 gm. The animals were housed in individual cages and fed *ad libitum*. The casein³ used in the experiment had been analyzed microbiologically by Stokes and associates ('45). For diet A (table 1) the quantities of the crystalline amino acids fed were the minimum levels suggested by Rose ('37), except that the quantity of leucine was reduced from 900 to 800 mg and of threonine from 600 to 500 mg per 100 gm of diet.⁴

RESULTS AND DISCUSSION

Table 1 shows the source of nitrogen fed each group, the nitrogen distribution in terms of the essential and non-essential nitrogen of casein and of the active and inactive isomers of the crystalline amino acids, the growth response, and the food consumption.

It is of interest that 50% of the nitrogen of the casein is that of essential amino acids and 50% is non-essential in character. The increase in percentage of casein is accompanied by an increase in non-essential nitrogen from that source and by a decrease in the quantity of inactive crystalline D-isomers. Although with the increase in casein content there is an in-

³SMA Vitamin Test Casein. Lot No. 14126.

⁴In accordance with suggestions made in a personal communication from Dr. W. C. Rose.

TABLE 1
Nitrogen per 100 gm of diet, protein equivalents, growth response and food consumption (20-day period)

DIET	CASEIN ¹		CRYSTALLINE AMINO ACIDS		TOTAL N	TOTAL ESSENTIAL N	TOTAL N MINUS INACTIVE D-ISOMERS	PROTEIN EQUIVALENT (N×6.25) ²	AVERAGE DAILY GAIN (20-DAY PERIOD)	AV. DAILY FOOD INTAKE	GAIN PER GM OF PROTEIN EQUIVALENT (N×6.25)
	Essential N	Non-essential N	Essential N	inactive D-isomers							
	mg	mg	mg	mg	mg	mg	mg	%	gm	gm	gm
A—Crystalline amino acids		...	788	195	983	788	788	4.93	0.8	7.3	2.2
B—2% Casein ³ + A.A.	147	146	641	158	1092	788	984	5.84	1.1	7.6	2.5
C—4% Casein + A.A.	293	293	498	117	1201	791	1084	6.78	1.7	7.2	3.5
D—6% Casein + A.A.	440	439	360	82	1322	800	1240	7.76	2.4	8.3	3.8
E—8% Casein + A.A.	586	586	229	54	1455	815	1400	8.78	3.9	9.5	4.7
F—1.28 × A.A.	1010	248	1258	1010	1010	6.32	1.2	6.7	2.8
G—1.77 × A.A.	1400	342	1742	1400	1400	8.78	1.2	4.6	3.0

¹ N content of casein 13.63%, air-dried basis.

² Total nitrogen minus nitrogen of inactive D-isomers × 6.25.

³ Calculated to dry basis.

crease in total nitrogen minus the inactive D-isomers of the crystalline acids, the quantity of essential nitrogen in the casein-containing diets was kept constant at the quantity provided by the minimum suggested levels of the essential amino acids, except for a slight excess at the 6% and 8% casein levels due to the arginine nitrogen supplied by casein. An increase in the quantity of the essential amino acids, when they were the sole source of nitrogen in the diet, provided a higher level of essential nitrogen. The protein equivalents ($N \times 6.25$) range from 4.93% for the diet containing the mini-

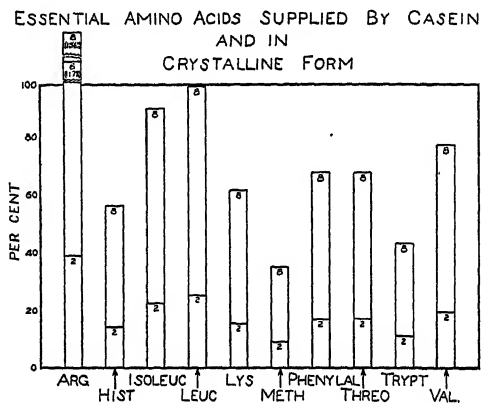


Fig. 1 For each amino acid the 100% line represents the requirements suggested by Rose ('37). The bars show the percentages of the amino acids supplied by the 2% and 8% levels of casein, the 4% and 6% levels being proportionately intermediate. The percentage of a crystalline acid is represented by the distance from the top of a bar to the 100% line.

mum amino acid levels to 8.78% for that made with 8% casein and added amino acids and that designed to provide 1.77 times the minimum amino acid requirement.

Figure 1 shows the percentages of the minimum levels of the essential amino acids supplied as casein and in the crystalline form. Except for arginine, none of the essential amino acids was supplied by casein in excess of the minimum requirement. When the level of casein was 8%, it was necessary to add only 0.8% of the leucine requirement as the crystalline acid, but for the other essential acids additions of the crys-

talline form ranged from 10.4% of the requirement for isoleucine to 65.2% of that for methionine. Higher levels of casein were not used, since the levels of several amino acids would have exceeded the minimum requirements.

When the minimum quantity of essential amino acids suggested as necessary for growth was fed (diet A), the average daily gain was 0.8 gm, which is what might be expected since the nitrogen of the diet is equivalent to only 4.93% protein. This response is slightly more than, but of the same order as, that reported by Kinsey and Grant ('44). It is not, however, in agreement with the report of Albanese and Irby ('43), who failed to obtain growth even when more than the minimum quantities of the essential amino acids were fed. An explanation of this difference in results has not been found, but the question may be raised as to whether the isoleucine content of the diet used by Albanese and Irby was adequate, since this amino acid was supplied as a mixture of leucine and isoleucine, leucine being provided also as a single amino acid.

At the 2% casein level the daily gain of 1.1 gm was not much better than that resulting when the crystalline acids were the sole source of nitrogen, but at 4%, 6% and 8% casein levels the growth responses were significantly greater and increased progressively with the increase in casein content until an average daily gain of 3.9 gm was obtained with diet E containing 8% casein. This is a very satisfactory rate of gain when it is considered that the diet contained nitrogen equivalent to only 8.78% protein, a quantity which would be equal in nitrogen content to 10.3% casein. The response of 3.9 gm is in close agreement with that of 3.4 gm obtained by Womack and Rose ('46) when they fed a mixture of 10 essential and 9 non-essential amino acids, although these authors did not report the quantities of amino acids used.

The progressive increase in daily gain is associated with an increase in non-essential nitrogen and a decrease in the quantity of inactive D-isomers, the quantity of essential nitrogen remaining practically constant. Whether the better gains

are due entirely to the nitrogen provided by the greater quantities of non-essential amino acids, or to this type of nitrogen along with some essential factor such as streptogenin (Woolley, '46), is as yet unknown. The close agreement with the response reported by Womack and Rose ('46) when crystalline acids were the sole source of nitrogen suggests, at least, that it is the nitrogen of the non-essential acids, rather than some essential factor, that is responsible for the gains shown on diet E. The question of whether certain of the non-essential amino acids are more necessary than others for the full effectiveness of the essential acids in promoting growth is one that has not yet been answered. Kinsey and Grant ('44) found, for example, that the addition of glycine to a diet containing the essential acids at the minimum required level failed to improve the growth rate, and Hier, Graham and Klein ('44) noted an inhibition of growth when glycine and L-proline were added separately to casein and fibrin diets.

It was necessary to use DL forms of three amino acids — isoleucine, threonine, and valine — whose D forms according to Rose ('38) are inactive for the rat. In diet A, the contents of inactive D-isomers per 100 gm of diet were 500 mg of isoleucine, 500 mg of threonine and 700 mg of valine. With the increase in the percentage of casein, the quantities of inactive isomers decreased until at the 8% level of casein the content of D-isoleucine became 52 mg per 100 gm of diet, of D-threonine 164 mg, and of D-valine 164 mg. Whether the inactive D-isomers have a retarding effect on growth, and their progressive decrease in the casein-amino diets allowed better growth, is not known. It should be noted, however, that for diets F and G, which contained the largest quantities of inactive isomers, growth was not depressed below that of diet A, which contained the next highest levels of these isomers. Thus, for diet F, the quantities of the inactive D-isomers of isoleucine, threonine, and valine per 100 gm of diet were 640 mg, 640 mg, and 896 mg, respectively, whereas for diet G the quantities were 885 mg, 885 mg, and 1239 mg.

The question of whether an excess of the essential amino acids, fed as the sole source of nitrogen, over the suggested minimum levels will supply the additional nitrogen necessary to promote better growth was given attention in the present investigation. Diet F, which contained 1.28 times the minimum suggested quantities of the essential amino acids, provided 1010 mg of essential nitrogen per 100 gm of diet, a value which falls between the total nitrogen minus the nitrogen of the inactive D-isomers of the 2% and 4% casein diets. Despite the fact that the 1010 mg of essential nitrogen is 28% greater than the 790 mg of these latter diets, the growth response is the same as that for the 2% casein diet. The factor 1.77 was used to provide a diet in which the total utilizable nitrogen would be 1400 mg per 100 gm of diet, the same as that in diet E which contained 8% casein and crystalline amino acids, and equivalent to 8.78% protein. Even with this increase in utilizable essential nitrogen the average daily gain was only 1.2 gm, which is no better than that noted when 2% casein and the essential acids (or 1.28 times the minimum amino acid levels) was the source of nitrogen. Similar observations have been made by Martin ('44), by Kinsey and Grant ('44) and by Hier, Graham and Klein ('44).

When the quantity of essential nitrogen was 815 mg and of non-essential 586 mg (diet E), excellent growth occurred. Diet F supplied 815 mg of essential nitrogen, which should meet the minimum requirements for good growth, and 585 mg of additional essential nitrogen in place of the non-essential nitrogen of diet E, yet poor growth resulted. Why this additional essential nitrogen is not utilized by the rat is a question of considerable interest.

The daily food consumption showed a progressive increase from 7.3 gm per rat per day for the animals on diet A to 9.5 gm for those on diet E, except in the case of diet C for which the value is not as high as would be expected. The increase in food consumption was accompanied by an increase in average daily gain on diets A to E, inclusive. The gain per gram of protein equivalent was calculated using the total

nitrogen per 100 gm of diet minus the inactive isomers times the factor 6.25 as the protein equivalent. The protein efficiency increased from 2.2 gm per gram of protein equivalent, for diet A, to 4.7 gm for diet E, containing 8% casein. The increase in protein efficiency is associated with increasing amounts of non-essential nitrogen provided by casein and by any unknown growth factors contained in it, and with a decrease in the content of inactive D-isomers.

For diet F the average daily food consumption was slightly less than that recorded for diet A, for which the food intake was the lowest of the series A to E; it was markedly less, 4.6 gm, for diet G. The protein efficiency for diets F and G, containing the larger quantities of crystalline essential acids, was somewhat higher, however, than that observed for diet A, which provided the suggested minimum quantities of these acids.

The question arises, of course, as to whether these lower food intakes were due to decreased palatability because of the larger quantities of crystalline acids. For diet F, 10.0 gm and for diet G, 13.9 gm of crystalline amino acids were present in 100 gm of diet. The larger of these quantities is considerably less than the 22.4 gm of the 10 essential and 9 non-essential crystalline acids per 100 gm of diet calculated for one of the diets fed by Rose and Fierke ('42), in which the average daily food consumption was 6.0 gm and the average daily gain 2.0 gm. Although lower palatability of diets F and G may have accounted in part for the lower food consumption, the larger quantities of crystalline acids used by Rose and Fierke did not reduce growth and food consumption below the levels obtained with these diets. The presence of the non-essential acids in the diet of Rose and Fierke suggests that they may be necessary for growth in conjunction with the essential acids. Furthermore, as has been pointed out previously, Womack and Rose ('46) more recently reported a gain of 3.4 gm per day when a mixture of 19 essential and non-essential acids were in the diet, although they did not state the quantities used.

SUMMARY

1. When the crystalline essential amino acids (supplied at the minimum levels suggested by Rose, '37) were the sole source of nitrogen, subnormal growth was obtained in the white rat.

2. Supplementation of 2, 4, 6 and 8% levels of casein with crystalline essential amino acids to meet the minimum suggested levels resulted in a progressive increase in growth rate, the highest rate, 3.9 gm per day, being noted at the 8% level of casein.

3. An increase in the quantity of essential nitrogen (supplied by the crystalline essential amino acids as the sole source of nitrogen) so that it was equal to the essential and non-essential nitrogen supplied by the 8% casein diet supplemented with crystalline acids, failed to cause an increase in growth rate significantly greater than that observed when the crystalline acids at their minimum levels were used.

ACKNOWLEDGMENTS

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STUDIES OF THE ALLEGED GROWTH PROMOTING PROPERTY OF VACCENIC ACID ¹

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Boer, Jansen and Kentie ('46, '47a) presented evidence in support of the view that the substance responsible for the superior growth promoting activity of summer butter is vaccenic acid, an 18 carbon chain fatty acid having a double bond between the 11th and 12th carbon atoms. The isolation and proof of structure of vaccenic acid were reported by Bertram ('28), who used margarine fat and butterfat as the source materials. The configuration was found to be trans, like that of elaidic acid, and therefore Bertram referred to it as iso-elaidic acid.

The reasoning which led Boer and his associates to test the growth promoting activity of this substance was unique. Earlier studies by Schantz et al. ('40b) as well as by Boer and others indicated that the growth promoting activity of butter-

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Some of the preliminary work involved in this research was carried out in co-operation with Dr. R. P. Geyer, now at Harvard University.

The work with synthetic vaccenic acid was made possible by the generosity of Dr. F. M. Strong and associates, who successfully synthesized both the cis and trans forms.

fat was present in certain fatty acid fractions. Distillation of the methyl esters of the butter fatty acids showed the activity to be present in the fraction containing 18 carbon atoms. The findings by Schantz et al. ('40a) that the fatty acid or acids which were responsible for the superior nutritive value of butterfat were present in the saturated fraction, and the observations of Boutwell et al. ('41) that hydrogenation of the saturated fraction decreased its nutritive value slightly, suggested that the effective agent might be an unsaturated acid whose lead soap was insoluble in alcohol, and that hydrogenation converted it into a saturated inactive compound. The fact that Boutwell et al. ('41) demonstrated improvement of the nutritional value of the unsaturated portion of butterfat following its hydrogenation can also be interpreted to mean that the same unsaturated compound was formed during hydrogenation of the original higher-unsaturated fatty acids.

All the above observations led Boer et al. ('46, '47a, '47b) to test the growth promoting activity of the only naturally occurring 18 carbon atom unsaturated fatty acid which forms a lead soap insoluble in alcohol.

The experimental evidence for the claim on behalf of vaccenic acid by Boer et al. lies in the finding that rats fed a ration containing butterfat grew better than those fed diets containing rapeseed oil, and that the addition of vaccenic acid to the latter diet at a level of 0.1% of the total ration caused the rate of growth to approach that of the animals fed butterfat. It was therefore concluded that this acid was one of the compounds in butterfat responsible for its superior nutritive value as compared with vegetable oils.

Work was started in this laboratory to determine the effect of supplementing our purified rations with vaccenic acid isolated from natural materials. The preliminary results were reported in the January 1948 issue of the Wisconsin Agricultural Experiment Station Bulletin (Geyer et al., '48).

More recently, Deuel et al. ('48) have reported on the alleged growth promoting activity of vaccenic acid. They observed no stimulatory effect on growth of young rats on either

rapeseed or cottonseed oil diets after administration of vaccenic acid or hydrogenated china wood oil. China wood oil has been reported by Boer et al. to be a good source of vaccenic acid.

Our experiments were carried out in order to study the problem further. We wished to determine whether vaccenic acid augments the growth of young rats when added to a purified diet containing corn oil, which under certain conditions has been shown to be inferior to butterfat. In this work we used vaccenic acid isolated from natural sources as well as that prepared by synthesis.

EXPERIMENTAL

The experiments were carried out with male weanling rats of the Sprague-Dawley strain, three weeks of age and with starting weights ranging between 40–45 gm. Unless otherwise stated, each group consisted of 6 rats weighed once weekly, and growth records were of 5 to 6 weeks' duration. The rats were housed in individual metal cages with raised screen bottoms and watered and fed daily ad libitum on diets the composition of which is given in table 1.

The butterfat used in these experiments was prepared by melting, decanting and filtering fresh sweet butter obtained from the University dairy. The corn oil used was a commercial product² of highest grade. Special precautions were taken with all fats to prevent rancidity and off flavors. All rations were mixed weekly and kept under refrigeration.

Two levels of water soluble vitamins were used in some of the experiments because it was previously reported that the difference in growth rates of rats fed butterfat or corn oil depends on the level of the B vitamins in the diet (Boutwell et al., '45).

Vaccenic acid was prepared according to the method of Bertram ('28). The same procedure was also employed by the Dutch workers. The final product we obtained was very simi-

² Mazola.

lar on the basis of iodine number and melting point to that of Bertram and Boer et al. We used these properties as the criteria for the purity of the material isolated from natural products. We believed that in our first 6 experiments the preparation used was not pure vaccenic acid, because it had

TABLE 1
Composition of the diets

BASAL MIXTURE					VITAMIN SUPPLEMENTS PER 100 GM OF BASAL MIXTURE		
Components	Diet						
	1	2	3	4	Medium level	High level	
	%	%	%	%	mg	mg	
Fat ¹	28	28	10 ²	28	Thiamine	0.20	0.50
Casein ³	20	20	5	..	Riboflavin	0.30	0.70
Salts ⁴	4	4	3	..	Pyridoxine	0.30	0.60
Sucrose	48	Ca pantothenate	1.50	3.0
Lactose	..	48	Choline	150.00	150.00
Rice (polished)	72	..	Folic acid	..	0.20
Yeast (dried)	10	..	Biotin	..	0.02
Skim milk powder ⁵	Niacin	..	0.60
(mineralized) ⁶	72	p-Aminobenzoic acid	..	15.00
					Inositol	..	50.00
					Carotene	0.56	0.56
					Tocopherol	2.24	2.24
					Calciferol	0.014	0.014
					2-Me-1,4-napthaquinone	0.21	0.21

¹ Butterfat or corn oil or corn oil containing 1% of vaccenic acid.

² Olive oil replaced corn oil in this experiment.

³ Extracted for three two-hour periods with boiling 95% alcohol.

⁴ Phillips and Hart ('36).

⁵ Extracted for 4 8-hour periods with diethyl ether.

⁶ Ten grams of the completed ration contain 0.6 mg each of copper sulfate and manganese sulfate and 12 mg of iron pyrophosphate.

an iodine number of 81.2 and melting point of 34°C. (Wiley method). Bertram ('28) reported I.N. 88 and melting point 38°C. We also observed that we could get purer material by increasing the number of recrystallizations from acetone at low temperatures. Butterfat was not a good source material because it contained only 0.8-0.9% of vaccenic acid, and therefore we looked for a richer source. A commercially available

hydrogenated fat,³ was found to be a good source of vaccenic acid (yield about 3%) and from this we could isolate larger amounts of purer material. The final preparation made from this source had an iodine number of 87.6 and melting point of 38°C. In certain experiments, the results of which are reported in table 3, this preparation was used.

Recently Ahmad, Bumpus and Strong ('48) synthesized both the *cis* and the *trans* forms of vaccenic acid. The melting point of the *cis* form of the acid was 5°C. and that of the *trans* isomer was 42–43°C. The iodine number and other physical constants of the compound as well as its chemical characterization by these workers, indicated the identity of its properties with those demanded theoretically for vaccenic acid.

The high melting point of the *trans* form of vaccenic acid has also been reported by Rao and Daubert ('48), and it is therefore obvious that the material isolated from natural sources by any other group of workers was in no case absolutely pure vaccenic acid.

The results of three experiments on the supplementary effects of vaccenic acid are given in table 2. It will be noted from these results that the differences in growth resulting from the butterfat and corn oil diets, even on medium levels of vitamins, were not significant. Furthermore, supplementation with vaccenic acid did not increase the growth of rats on the corn oil diets. Of the 6 groups of animals fed supplements of vaccenic acid, only one showed a slight increase in growth, which, however, was within the experimental variation generally observed in biological studies of this type. In other cases the acid appeared to have a slightly depressing effect on the growth of the animals. No other explanation, except possibly biological variation, could be held responsible for this effect because chemical examination did not indicate the presence of lead, chlorine or other contaminating material in the vaccenic acid preparations.

On the basis of previous reports from this laboratory (Boutwell et al., '43) that the difference between butterfat and

³ Crisco.

corn oil is considerably greater when lactose is the carbohydrate in the diet, it was decided to repeat the experiments with this carbohydrate replacing sucrose. Two such experiments with lactose (diet no. 2) were carried out. In both the superiority of butterfat over corn oil on a lactose diet was again demonstrated. On the other hand, the vaccenic acid was found to have no effect as a supplement to corn oil diets even under these conditions.

TABLE 2

Effects of additions of vaccenic acid to corn oil diets

(Figures represent the average number of grams gained, range is shown by numbers in parentheses. Six rats in each group)

CARBO- HYDRATE USED	DIET NO.	EXPT. NO.	NO. OF WEEKS	VITAMIN LEVEL	BUTTER- FAT	CORN OIL	CORN OIL + VACCENIC ACID
Sucrose	1	1	6	Medium	173 (162-187)	175 (149-216)	174 (171-186)
				High	197 (178-220)	186 (162-201)	190 (165-225)
Sucrose	1	2	5	Medium	160 (145-188)	153 (147-163)	150 (126-192)
				High	174 (160-188)	156 (149-164)	150 (140-160)
Sucrose	1	3	5	Medium	159 (129-201)	153 (130-186)	141 (127-149)
				High	170 (141-191)	187 (176-198)	178 (131-212)
Lactose	2	4	6	Medium	130 (115-145)	122 (103-134)	122 (112-143)
				High	147 (138-158)	143 (128-158)	138 (128-150)
Lactose	2	5	6	Medium	161 (127-200)	128 (114-154)	126 (116-145)
				High	154 (140-189)	141 (123-152)	129 (105-152)
Polished rice	3	6	5		166 (160-176)	163 ¹ (150-174)	154 ² (127-185)

¹ Olive oil.

² Olive oil containing 1% vaccenic acid.

After failing to observe on our purified diets any growth promoting effects of vaccenic acid, it was decided to determine whether this activity of the material could be obtained with a natural diet, as closely resembling that used by Boer et al. ('46, '47a, '47b) as possible. The diet used by them consisted of 72% polished rice, 5% extracted casein, 10% dried yeast, 3% salt mixture and 10% fat (butterfat or rapeseed oil or rapeseed oil supplemented with vaccenic acid). The diet we used differed from theirs only in the replacement of the rapeseed oil with olive oil. We made our initial trials with rapeseed oil obtained in the local market but due to its poor quality, mostly due to rancidity, we decided not to use it.

Again 6 male rats were placed in each group; their average 6-week weight gain in grams is indicated in table 2, experiment 6. Here again no stimulatory effect of vaccenic acid could be detected. The growth of the animals on butterfat and olive oil diets was also found to be nearly equal. It should be emphasized that the starch of polished rice was the main carbohydrate in the diet, and this confirms the report of Boutwell et al. ('43) that when starch is the dietary carbohydrate there is no difference between the growth promoting activity of butterfat and that of the vegetable fats studied.

As mentioned before, all the above experiments were carried out with weanling three-week old male rats obtained from the Sprague-Dawley Company. On the assumption that weanlings might carry from their mothers a store of vaccenic acid and therefore obliterate the possible beneficial effect of feeding this acid, we thought it desirable to test the activity of vaccenic acid by using young rats depleted of this material. With this in mind, we depleted young female rats (future mothers of the experimental weanlings) by keeping them on diet no. 1 with corn oil as the dietary fat. This was done in view of our previous finding that body fat obtained from rats fed a purified diet containing corn oil as the only dietary fat contained no vaccenic acid (Geyer et al., '47b). For purposes of comparison we also used weanlings from mothers on diet no. 1 with butterfat as the source of fat. In addition, two groups of

females were placed on a skim milk powder diet (table 1, diet no. 4), one group receiving butterfat, the second corn oil.

When the females matured they were mated with normal males. Their young were selected in groups of three weanling litter mates of the same sex and weight and placed on three diets: one on butterfat, the second on corn oil, and the third on corn oil containing 1% vaccenic acid. The young from the sucrose-fed mothers were placed on purified sucrose diets, while those from the skim milk mothers were placed on skim milk diets. Table 3 gives the results.

TABLE 3

*Effects of vaccenic acid supplementation on corn oil diets of depleted young
(Figures represent the average number of grams gained in 6 weeks;
range is shown by numbers in parentheses)*

SERIES	DIET OF THE MOTHER	SEX	NO. OF TRIOS OF LITTER MATES	BUTTERFAT	CORN OIL	CORN OIL + VACCENIC ACID
I	Butterfat mineralized skim milk	M	6	126 (104-151)	110 (97-119)	105 (91-118)
II	Corn oil mineralized skim milk	M	9	148 (122-165)	126 (110-148)	126 (109-151)
III	Butterfat-sucrose	M	7	136 (120-159)	122 (102-140)	131 (103-152)
IV	Corn oil-sucrose	M	9	125 (105-142)	121 (108-138)	116 (98-141)

It was again observed that vaccenic acid produced practically no beneficial effect on the rate of growth of young rats. Only in series 3, where the young were obtained from butterfat-sucrose-fed mothers, was the average growth of the group supplemented with vaccenic acid higher than that of the unsupplemented group. But even here a stimulatory effect was observed in only 4 out of the 7 groups of animals employed in the series, while in the other three groups no effect was noticeable. Considering the individual variations often

observed among animals in an experiment of this type, it is doubtful whether these higher values are significant.

In view of the fact that we always had some doubt about the purity of the preparation isolated from natural sources, we decided to reinvestigate the problem by using synthetic vaccenic acid, prepared in this laboratory by Ahmed, Bumpus and Strong ('48). In our first trial we used the *cis* form of the synthetic compound at a level of 1% of the total fat administered. The plan of the experiment was identical with

TABLE 4

*Effects of additions of synthetic vaccenic acid on the growth of young rats
(Six animals in each group)*

FAT	FORM OF VACCENIC ACID	LEVEL OF VACCENIC ACID	AVERAGE GAIN IN WT. IN 5 WKS.	
			Expt. I	Expt. II
		% in fat	gm	gm
Butterfat	. .	.	107	107
Corn oil	90	88
Corn oil supplemented with vaccenic acid	<i>cis</i>	1.0	87	80
Corn oil supplemented with vaccenic acid	<i>cis</i>	0.3		75
Corn oil supplemented with vaccenic acid	<i>cis</i>	0.1		86
Corn oil supplemented with vaccenic acid	<i>trans</i>	1.0		85

that described earlier in connection with experiments 4 and 5 (table 2). Lactose was used as the sole carbohydrate of the diet, and the medium level of the B vitamins was supplied (table 1). The results are given in table 4.

Although over a period of 5 weeks the weight gains of the animals on the supplemented or unsupplemented diets were exactly equal for some unexplained reason, in the second and third week the average growth of the group receiving vaccenic acid was somewhat better than that of their controls. In order to check the possibility that the amount of vaccenic acid which we were administering to the animals was too high and was

possibly producing some undesirable effect, we decided to lower the level of vaccenic acid in the diet to $\frac{1}{3}$ and $\frac{1}{10}$ of the amount we gave before. It should be mentioned that Boer and his group ('46, '47a, '47b) used 0.1% of vaccenic acid in their diets, but in practically all of our experiments we used the vaccenic acid at a level of 0.28% of the total diet.

At this time the trans form of the synthetic vaccenic acid was also made available and its activity tested. Table 4 gives the results with both the cis and trans forms at different levels of intake. No stimulatory effect of either form of vaccenic acid at any level could be observed.

DISCUSSION

Under our experimental conditions the work described demonstrated conclusively that the rate of growth of rats is little affected whether the diet contains vaccenic acid or not. This was found to be true whether the basal diet contained as the main carbohydrate sucrose, lactose or starch, or the dietary fat used was corn oil or olive oil, or whether the levels of the water soluble vitamins were medium or high. Neither were there any differences when vaccenic acid was isolated from natural fats or made by synthesis. The results also confirm the report of Boutwell et al. ('43) that butterfat is always superior to corn oil when lactose is the sole carbohydrate of the diet.

Recent studies by Jack and Hinshaw ('47) on the nutritive value of fractions obtained by cold crystallization of milk fat also indicate that some other factor or factors must be responsible for the superior growth promoting activity of milk fat. These authors reported the -53°C . filtrate fraction to have a growth promoting action much superior to that of the milk fat itself. In view of the high melting point of vaccenic acid it would appear that after crystallization at -20°C . all of this compound would be left in the precipitate, yet the growth gain and efficiency of utilization of the diet on this fraction (-20°C .) as the source of dietary fat were the least of the group when the diet was compared with those contain-

ing the original milk fat and the other fractions. The existence of such a fraction, having growth stimulating properties superior to the original butterfat, has also been observed in our recent studies on the nutritive value of a fraction of butterfat prepared by cold crystallization (Geyer et al., '47a; Nath et al., '48).

The negative effect of vaccenic acid on the growth of rats also became apparent in our recent studies on the comparative nutritive values of commercial hydrogenated cottonseed oil⁴ and corn oil (unpublished data). In spite of the fact that we can isolate from the hydrogenated product about 3% of material presumably corresponding to vaccenic acid, this fat did not support better growth of young rats than did corn oil.

From all these experiments it is obvious that vaccenic acid has no special growth stimulating properties. The fact still remains that there is something present in butterfat at certain seasons of the year which may be either a definite chemical compound or, more likely, a fraction rich in certain favorable fatty acids. This factor or factors concentrated by cold fractionation from acetone has greater growth stimulating properties than corn oil or the butterfat itself.

SUMMARY

1. No increase in the growth of young rats on diets containing either corn oil or olive oil resulted from supplementing the diets with vaccenic acid isolated from either butterfat or commercial hydrogenated cottonseed oil.

2. Replacing the sucrose by lactose or starch (rice) as the source of carbohydrate in the diets, or changing the levels of the water soluble vitamins, did not alter the results as far as the vaccenic acid supplementation was concerned.

3. Similar results were obtained when weanling rats from depleted mothers were used.

4. Supplementing a corn oil-lactose diet with either the *cis* or *trans* forms of synthetic vaccenic acid produced no growth stimulating effects.

⁴ See footnote 3, page 765.

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CALORIC RESTRICTION AND PROTEIN METABOLISM IN THE GROWING MOUSE¹

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SEVEN FIGURES

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It is a well-established principle of nutrition that restrictions in caloric intake below certain minimum requirements result in a decreased retention of nitrogen. For example, Deuel ('48) cites evidence that in man a maximum protein-sparing effect of non-protein calories may be noted with intakes of 1500 calories per day but not when the caloric level is reduced to 600 calories per day. In the growing animal it is possible to adjust caloric intake and to measure nitrogen retention with great accuracy. Employing rats and mice, Bosshardt et al. ('46a) have presented evidence that with increasing restriction of calories protein utilization remains constant until a critical caloric intake is reached. Restriction beyond this point results in a very rapid diminution in the efficiency of protein retention. Benditt et al. ('48) have shown that this same phenomenon is apparent in the protein-deficient rat during a period of repletion. In fact, the critical caloric level for optimum protein utilization in the rat was essentially identical in both of these studies.

Deuel ('48) has reviewed a number of investigations which show that the protein-sparing effect of carbohydrate is considerably greater than that of fat when subjects are ingest-

¹ A preliminary report of this data was presented before the American Institute of Nutrition, Chicago, May 22, 1947.

ing the same quantities of protein. Furthermore, in complete protein starvation carbohydrate, but not fat, has the property of sparing body protein. Conclusions reached by various investigators (Landergren, '03; Best and Taylor, '39) make it appear logical that protein-sparing can be related to three different metabolic pathways: (1) Protein may act as a precursor of glucose and thus be diverted into the formation of essential carbohydrate in complete or severe dietary carbohydrate deprivation; (2) intermediary products of carbohydrate metabolism may be available for the resynthesis of amino acids by utilizing some of the nitrogen released by the catabolism of protein; (3) both dietary fat and carbohydrate supply essential calories and in their absence protein must be diverted to the production of calories.

In considering the above factors that may regulate protein-sparing, it would seem likely that equal caloric intakes of fat or carbohydrate would spare protein to the same extent if some quantity of carbohydrate were present in the diet. Allison, Anderson, and Seeley ('46) maintained dogs on a markedly restricted caloric intake and found, as was to be expected, that the nitrogen balance index decreased; fat or carbohydrate added to the calorically deficient intake increased the nitrogen balance index to the same extent.

The studies presented here were designed to investigate the growth promoting effects of fat, carbohydrate and protein. All were conducted on the growing mouse receiving caloric intakes that were known to be below the minimum level for optimum protein utilization.

EXPERIMENTAL METHOD

Food utilization was studied with growing mice throughout a wide range of caloric intakes. Two types of feeding were employed, *ad libitum* and restricted. Two protein sources, casein² and wheat gluten, were used. Although there is an appreciable difference in their nutritive qualities, both sources

² Borden's Labco.

are able to support good growth in the mouse if sufficient protein is fed.

The basal diet in the *ad libitum* experiments consisted of 2% corn oil³, 20% glucose⁴, 4% salt mixture (Hubbell, Mendel and Wakeman, '37), 2% cellulose⁵, and 1% Wilson's 1:20 liver concentrate powder, and was supplemented so that each 100 gm of diet contained 4 mg of α -tocopherol, 900 U.S.P. units of vitamin A, 180 U.S.P. units of vitamin D, 1 mg of 2-methyl-1,4-naphthoquinone diacetate, 0.8 mg of thiamine hydrochloride, 1.6 mg of riboflavin, 0.8 mg of pyridoxine hydrochloride, 4.0 mg of niacin, 4.4 mg of calcium pantothenate, 4.0 mg of para-aminobenzoic acid, 200 mg of choline chloride, and 21.6 mg of inositol. The remainder consisted of the protein source, hydrogenated cottonseed oil⁶ and white dextrin, the levels being varied to obtain diets with different protein levels and caloric densities.

Diets with varying protein levels and caloric densities, using casein and wheat gluten as the protein sources, were fed *ad libitum* to groups of 7 male albino weanling mice (Sharp and Dohme, Swiss Webster strain) for 10-day periods. Records of the body weight gains and of the food and protein intakes of the test animals were kept. Only the data obtained from those groups in which the average protein intake corresponded to that giving maximum utilization with isocaloric diets were considered in this report. Intakes giving maximum utilization were 0.19 gm of casein per mouse per day and 0.60 gm of wheat gluten per mouse per day (Bosshardt and co-workers, '46b).

In the restricted feeding experiments the daily protein intakes per mouse were maintained at 0.19 gm of casein and 0.60 gm of wheat gluten. The daily intakes of the vitamins, minerals, and roughage were also constant and were the amounts present in 2 gm of the basal diet. An intake of 2 gm per mouse per day is the average intake of the basal diet

³ Mazola.

⁴ Cerelose.

⁵ Cellu flour.

⁶ Primex.

containing 10% casein and 25% fat under conditions of ad libitum feeding for a 10-day period. Differences in caloric intake were obtained by varying the daily intakes of fat and white dextrin.

For the calculation of caloric intakes the constants employed were 9.3 cal. per gram of fat, 4.0 cal. per gram of wheat gluten, 4.4 cal. per gram of casein, 3.75 cal. per gram of glucose, and 4.23 cal. per gram of dextrin. Caloric intakes were calculated on the basis of the average body weight $^{2/3}$. This was as-

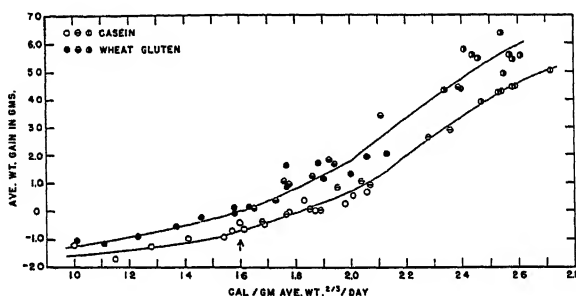


Fig. 1 The relationship between body weight gain and caloric intake with the two protein sources, casein and wheat gluten. The graphic symbols used are \odot and \bullet for ad libitum feeding, \odot and \bullet for increases in caloric intake by increasing intake of carbohydrate from \uparrow , and \ominus and \ominus for increases in caloric intake by increasing intake of fat from \uparrow .

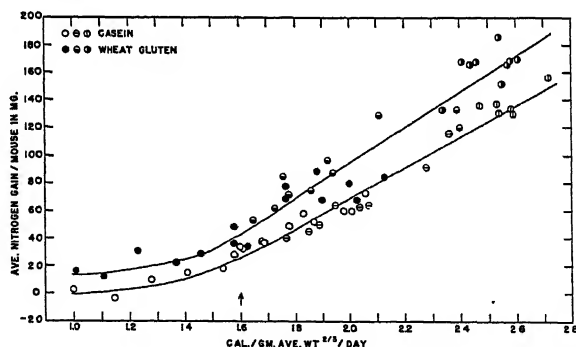


Fig. 2 The relationship between body nitrogen gain and caloric intake with the two protein sources, casein and wheat gluten. The graphic symbols used are \odot and \bullet for ad libitum feeding, \odot and \bullet for increases in caloric intake by increasing intake of carbohydrate from \uparrow , and \ominus and \ominus for increases in caloric intake by increasing intake of fat from \uparrow .

sumed to be proportional to average body surface area during the 10-day period.

RESULTS

When the average weight gains or body nitrogen gains were plotted against the caloric intakes per unit of body surface area, it was found that at any given caloric intake the animals receiving the wheat gluten grew better than did those receiving casein (figs. 1 and 2). Each point in the figures represents the average of 7 mice. A similar relationship was found when the caloric intake per unit of body surface area was plotted against the "caloric efficiency ratio" (gm gain in weight per cal. consumed) or against the body calorie equivalent based on the fat and protein contents (figs. 3 and 4).

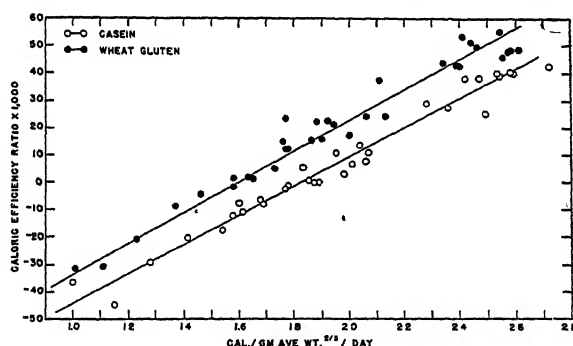


Fig. 3 Relationship between caloric intake per unit body surface area and the "caloric efficiency ratio" with mice fed casein and wheat gluten.

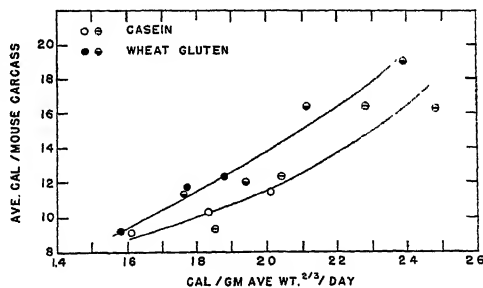


Fig. 4 Relationship between caloric intake per unit body surface area and the body calorie content based upon the fat and protein contents. ○ and ● indicate caloric intake increases by additional carbohydrate and ⊖ and ⊙ by additional fat.

The protein ($N \times 6.25$), fat, and water contents accounted for 94 to 96% of the total body weight. In no case was there a difference between the effects of fat or carbohydrate, the response per calorie being the same by all methods of calculation.

As the caloric intake was decreased, the difference in the nutritional quality of the two proteins was minimized (fig. 5). However, only a very small fraction of the ingested protein was used for tissue synthesis when the caloric restriction was severe. It would appear that throughout the range of caloric

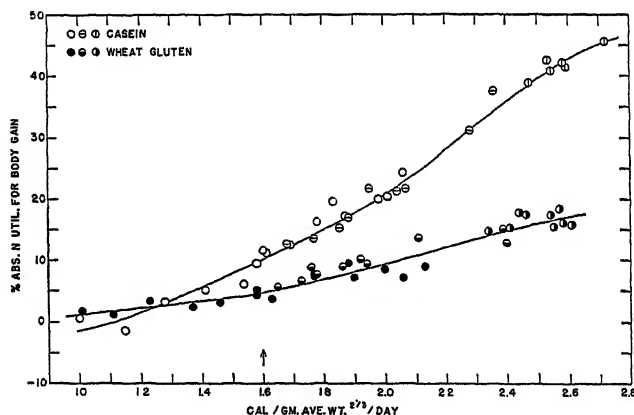


Fig. 5 The relationship between the caloric intake per unit body surface area and the growth utilization of dietary protein expressed as the percentage of absorbed nitrogen utilized for body nitrogen gain.

intakes studied differences in growth were due more to the quantity of protein supplied than to the nutritive quality of that protein.

In order to test this point, a study was made in which a portion of the fat and carbohydrate in the casein diets was replaced with equicaloric amounts of casein or wheat gluten, so that the protein intakes of the test animals were the same as in the wheat gluten series (0.60 gm per mouse per day). These replacements were made in the diets supplying 5.5 to 7.0 cal. per mouse per day. This range is approximately 50 to 65% of the caloric intake when feeding is ad libitum.

This replacement of non-protein calories resulted in an increase in growth and body nitrogen gain to that previously obtained with the wheat gluten feeding (figs. 6 and 7). The effects were the same when the additional protein was either casein or wheat gluten.

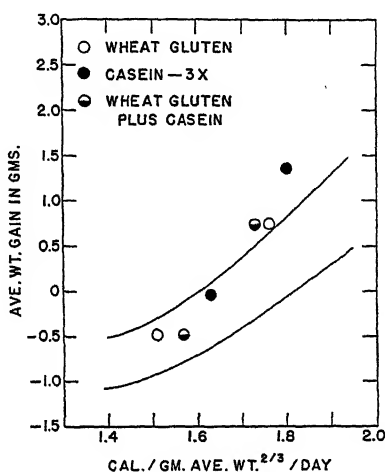


Fig. 6 The effect of replacing non-protein calories with protein on the weight gains of mice subjected to a caloric restriction. The two lines are portions of the curves shown in figure 1.

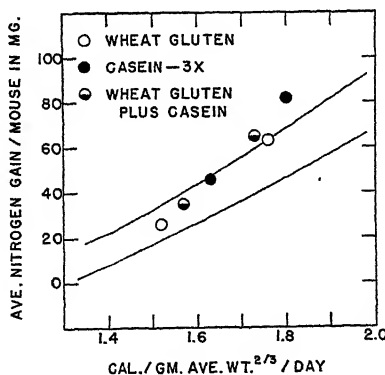


Fig. 7 The effect on the body nitrogen gains of mice subjected to a caloric restriction of replacing non-protein calories with protein. The two lines are portions of the curves shown in figure 2.

DISCUSSION

The major factor limiting growth throughout the wide range of caloric intakes studied in the present investigation was the amount of protein ingested. At the level of adequate caloric consumption amounts of wheat gluten and casein were fed that would give optimum efficiency of protein utilization for growth. These levels of intake were not adequate for an optimum growth rate for either of the proteins studied. If the intake of casein was increased three-fold so as to equal the intake of wheat gluten, percentage utilization of the casein dropped from approximately 46 to 24 (unpublished data). This means that protein utilization was roughly halved by increasing the intake three times and, therefore, additional protein deposition must have taken place. When a restriction in caloric consumption was imposed so that approximately half of an adequate caloric intake was being supplied (1.6 cal./gm ave. wt. %/day), the same general relationship held. A three-fold increase in protein intake (casein) caused a decrease by one-half in the efficiency of utilization. Again extra protein deposition resulted, in spite of the fact that in this case there was a concurrent deficiency in calories.

As the severity of caloric restriction was increased the decrease in efficiency of utilization of the protein from the two sources appeared to proceed at different rates, so that at very low caloric intakes the percentage of absorbed nitrogen utilized for body gain became the same for both proteins. This method of expressing protein utilization is complicated by the fact that wheat gluten was consumed in an amount three times greater than that of casein. On the basis of body nitrogen stored, the two protein sources maintained approximately the same relationship to one another throughout the entire range of caloric intakes. When caloric restriction was severe, the percentage utilization of protein became so low that minor differences in the nutritive values of different proteins were no longer of major importance and the over-all effect of protein nutrition was governed primarily by the quantity of protein ingested.

The conclusion seems justified that in conditions of calorie undernutrition the efficiency of protein utilization is governed largely by the extent of the caloric restriction, and that within certain limits increased amounts of protein can be utilized by the body if the protein intake is increased, even though no change in calorie consumption is effected. If caloric consumption is increased, protein will be utilized by the body to a greater extent, even though the protein intake is held constant. However, if calories are increased by adding protein rather than non-protein nutrients, the amount of protein utilized will be still larger. This means that in many states of semi-starvation protein is the most important limiting factor.

From the data given in figure 4 it was possible to calculate the approximate changes in energy expenditure that accompanied restriction in caloric intake. It was estimated that with a 34% decrease in energy intake (2.39 to 1.53 cal./gm ave. wt.%/day) there was a 14% decrease in energy expenditure (21.6 to 15.7 cal./gm ave. wt.%/day). This shows that in the growing animal on a constant protein intake energy conservation is accomplished by a reduction in basal metabolism. This, of course, has been observed in semi-starvation by many investigators.

Another point of interest is the observation that fat and carbohydrate are equal in their protein-sparing effect. It must be kept in mind that some carbohydrate was present in all diets, so this observation in no way contradicts earlier studies showing a complete lack of protein-sparing by fat under conditions of total carbohydrate deprivation. In addition, relatively large amounts of protein were present in all diets. As the caloric restrictions became more severe, increasing amounts of protein were catabolized and thus were undoubtedly available for conversion into carbohydrate.

The results obtained in this study elaborate and confirm the results of Elman, Davey, and Kiyasu ('45), who found that positive nitrogen balance could be maintained on a low

caloric intake if sufficient protein were fed, but not when a portion of the protein was replaced by carbohydrate.

SUMMARY

The effects of restricting the caloric intake by decreasing the consumption of fat and carbohydrate while holding constant the protein, vitamin, and mineral intakes have been studied in growing mice. Under such conditions there were observed: (1) a decrease in growth rate; (2) a decrease in the efficiency of protein and calorie utilization for growth; and (3) a decrease in energy expenditure.

Extra quantities of dietary protein caused increased growth to approximately the same extent in mice receiving an adequate caloric intake or a caloric intake restricted to about one-half the adequate level.

With low caloric intakes, extra calories in the form of protein caused a much greater growth response than equivalent calories supplied as fat or carbohydrate.

Under the conditions of this study, in which all diets contained some fat, carbohydrate, and protein, fat and carbohydrate were equal in their protein-sparing effect.

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THE EFFECTS OF ORAL ADMINISTRATION OF DIFFERENT PROTEINS ON THE PLASMA PROTEINS OF PROTEIN-DEPLETED DOGS

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INTRODUCTION

The nutritive quality of a protein can be measured biologically in a variety of ways, among which the growth method of Osborne and Mendel ('26), the biological value method of Mitchell ('44), and the nitrogen balance index method of Allison and Anderson ('45) are the most commonly used. Madden and Whipple ('40) believe that the depletion of plasma proteins puts a strain upon the body and accentuates the normal demand for plasma protein production, perhaps even at the expense of building other tissue proteins. This hypothesis was utilized by them and by other investigators (Cox and Mueller, '44; Holman et al., '34; Melnick et al., '36a; Seeley, '45; and Weech and Goettsch, '38a) as a basis for the measurement of the nutritive values of proteins in terms of their ability to stimulate the production of plasma proteins in standardized dogs depleted of proteins. The results of their studies reveal that not only do various proteins differ in their ability to regenerate plasma protein, but some favor the production in the plasma of more globulins than albumin. The latter view was substantiated by the re-

sults (Chow et al., '48) of electrophoretic analysis of the plasma of protein-depleted dogs made before and after protein hydrolysate feeding. Such a study permits a more complete analysis of the globulin fractions and a more precise determination of the albumin. The results indicate that lactalbumin hydrolysate feeding stimulates the regeneration of albumin but not of globulins, whereas casein hydrolysate promotes the production of both albumin and globulins.

The above study was extended to include the oral feeding of 5 whole proteins commonly present in our daily diets, in order to ascertain whether whole proteins, like the hydrolysates, also differ both qualitatively and quantitatively in their ability to regenerate different components of plasma proteins. Tryptic digests of casein and lactalbumin, whose chemical and biological properties were described by Bolling et al. ('47), were included in order to ascertain whether a hydrolysate which presents to the body for tissue synthesis a whole pattern of amino acids and peptides, available at one time, might not be superior to the whole proteins, which must be digested and then synthesized. Lastly, it is of interest to ascertain whether the same proteins are qualitatively similar when different methods of measurement are used. The results of our investigation are presented in this paper.

EXPERIMENTAL

Depletion of the protein reserve in dogs

Normal animals can be depleted of their protein reserves by plasmapheresis, by prolonged protein-free feeding, or by feeding a diet with inadequate caloric intake but an otherwise sufficient amount of nutritionally adequate protein to maintain nitrogen balance. In the last situation (Lusk, '28; Elman, '44), protein is used primarily to supply the necessary energy. Any one of the above procedures, or a combination of them, should provide us with a means of protein depletion. Therefore, attempts were made to so deplete three groups of healthy normal dogs of their protein reserves, in order to

ascertain whether protein depletion, regardless of its cause, will result in similar changes in the electrophoretic patterns of the plasma proteins, and in the total circulating plasma proteins (plasma volume \times plasma protein concentration). The procedures used for the estimation of plasma volume, total plasma protein concentration, and electrophoretic analyses have already been described by Chow, Seeley, Allison, and Cole ('48).

The first group of dogs (Group A), totaling 15 animals, was kept on a protein-free diet and plasmapheresed once a week by withdrawing approximately one-fourth of the total blood volume calculated on the basis of their body weight. The blood was citrated and centrifuged and the plasma was removed. The red cells were washed once with a sterile 0.85% sodium chloride solution and suspended in a normal saline solution equal in volume to that of the plasma removed (Seeley, '45). The suspension was returned to the animals intravenously. Plasmapheresis was repeated as often as necessary in order to bring the plasma protein concentration to about 4.5 to 5.2 gm %. Its frequency was dependent on the adequacy of the initial protein reserve.

The second group of 15 normal animals was put on a protein-free diet calculated to supply 80 cal. per kg body weight per day. The composition of the diet was that used by Allison and Anderson ('45). In our experience, this procedure of depletion required from 6 to 8 weeks to lower the plasma protein concentration to the desired level. A third group of 5 animals was put on a casein diet containing 0.20 gm of casein nitrogen and 30 cal. per kilogram body weight per day. This diet was prepared by replacing sucrose in the protein-free diet with casein. Two hundred milligrams of casein nitrogen were sufficient to keep normal dogs with adequate protein reserves in nitrogen balance, provided no less than 60 cal. per kg body weight per day were supplied. To deplete dogs on this partial starvation diet required three to 4 weeks.

The results (see table 1) of analyses of the composition of plasma protein before and after such depletion demon-

strate that each of the three methods of depletion brought about a decrease in plasma volume and plasma protein concentration, and therefore total circulating plasma proteins, by 35 to 40%. In terms of percentages the outstanding changes were a sharp decrease in albumin and a marked increase in the alpha globulins. In terms of the circulating plasma proteins, the albumin decreased markedly and the gamma globulin tended to decrease slightly; whereas the alpha globulins changed but little or were slightly increased.

TABLE 1

Composition of plasma protein of dogs before and after protein depletion by different methods

METHOD OF DE- PLETION	NO. OF DOGS USED	TREAT- MENT	PLASMA VOLUME	PLASMA PROTEINS				TOTAL CIRCULATING PLASMA PROTEINS			A/G	
				Total	Fractions			Albu- min	Globulins			
					Albu- min	Globulins			Albu- min	Globulins		
						$\alpha_1 + \alpha_2$	γ			$\alpha_1 + \alpha_2$		γ
			ml	gm%	%	%	%	gm	gm	gm		
A ¹	15	Control	473	6.21	42	16	9	12.4	4.5	2.5	0.75	
	15	Depleted	427	4.48	21	26	9	4.0	4.9	1.8	0.27	
B ²	15	Control	603	6.74	40	16	8	16.0	6.6	3.0	0.67	
	15	Depleted	510	5.04	21	26	11	5.3	6.8	3.0	0.27	
C ³	5	Control	500	6.37	40	15	9	12.7	4.8	2.9	0.67	
	5	Depleted	425	4.52	21	25	10	3.8	4.5	1.6	0.27	

¹ By plasmapheresis and protein-free feeding.

² By protein-free feeding alone.

³ By feeding a diet low in calories.

Since any one of the three methods gave essentially the same results, the protein-free feeding method without plasmapheresis was chosen for further experiments because of its convenience and the minimum exposure of the animals to possible infections during plasmapheresis. In order to standardize our depleted dogs as much as possible, the protein-free feeding was continued only until the albumin to globulin ratio levelled to 0.2 to 0.3. Most animals did not survive further depletion. We believe that these dogs were as severely depleted as if they had also been plasmapheresed.

Chemical and nutritive properties of test proteins

Five commonly consumed proteins of varying nutritive quality (whole egg, egg white, lactalbumin, casein, and wheat gluten) were used for the repletion studies. Enzymatic digests of casein and lactalbumin were also included for comparison with the respective undigested proteins.

TABLE 2

The chemical and biological properties of the proteins and the protein hydrolysates used

SUBSTANCES	N	DEGREE OF HYDROLYSIS ¹	NITROGEN BALANCE INDEX ²	MAXIMUM PROTEIN EFFICIENCY ^{3,4}	CANNON RAT DEPLE- TION ^{4,5}
	%				
Egg white	15.0	0	1.1	3.3	6.4
Lactalbumin	13.0	0	1.0	3.2	5.8
Whole egg	11.8	0	0.95	1.8	5.6
Casein	13.8	0	0.8	2.3	5.5
Wheat gluten	13.2	0	0.3	0.7	2.9
Lactalbumin hydrolysate	12.7	35	1.0	3.1	..
Casein hydrolysate	13.5	25	0.8	2.2	..

¹ Degree of hydrolysis = increase in amino nitrogen (the nitrous acid method) calculated as per cent of the total nitrogen.

² Nitrogen balance indexes were determined in normal dogs by the method of Allison and Anderson ('45).

³ Protein efficiency was determined in mice using the method of Osborne and Mendel ('26). The mice were fed at 5 levels of nitrogen intake.

⁴ The authors are indebted to Dr. A. Black for these measurements.

⁵ Proteins were fed to rats depleted of protein reserves according to the procedure of Cannon ('45). Protein efficiency was measured by determining the weight gain of these animals per gram of food nitrogen consumed.

Since these test proteins were not chemical entities and their chemical composition and nutritive value might be subject to variation in commercial processing, a few of their chemical and biological properties are shown in table 2. The results demonstrate that with respect to nitrogen balance index, a measure of the amount of protein nitrogen necessary to

maintain nitrogen balance in normal dogs, and the growth efficiency, a criterion of ability to support growth in normal mice or to replace body tissues lost due to depletion (Cannon's technique, '45), these 5 proteins and the two protein hydrolysates are, as is commonly believed, in the following order of value: egg white and lactalbumin, whole egg, casein, and wheat gluten. The hydrolysates of lactalbumin and casein have the same nutritive values as their original proteins.

The liver protein regeneration properties of these proteins were also determined by us using the Harrison and Long ('45) procedure. However, we found that the caloric intake must be sufficient; otherwise the results were not reproducible. The following procedure, which yielded satisfactory results, is given in detail:

Healthy normal rats of either sex, weighing approximately 225 gm, were fed ad libitum a stock diet consisting of casein, corn, starch, and mazola with adequate vitamin and salt supplements. A week prior to the experiments these rats were weighed every other morning. Only those animals which showed constant weights (± 3 gm) within this period were pooled. Ten rats, the control group, were sacrificed at this time. The remaining animals were given only water ad libitum for 48 hours in order to deplete their liver proteins. Ten of these animals (the fasted group) were sacrificed. The remaining animals were divided into groups of 10 each and were then fed test diets containing 40% protein for 4 successive days (10 gm of diet for the first day and 8 gm for the next three days). It was found that, as a rule, the rats consumed all the rations as scheduled; animals that did not were discarded. The composition of the diet was 40% test protein, 22% a partially hydrolyzed starch preparation,¹ 8% yeast, 4% salt, 2% codliver oil, 24% hydrogenated cottonseed oil.² Twenty-four hours after the last feeding the animals were anesthetized with nembutal and exsanguinated. Immediately after sacrifice, the livers were washed with a normal saline solution until

¹ Amidex. Corn Products Refining Company, New York, N. Y.

² Primex.

free from red cells and were dried on filter paper and weighed. The liver was then homogenized with about 70 ml of 0.85% NaCl solution in a micro Waring Blendor and made up to a volume of 250 ml. Aliquot samples (1.0 ml) were taken for determination of the total nitrogen by the micro Kjeldahl method.

The results of a typical experiment (see table 3) demonstrate that our samples of lactalbumin and casein were defi-

TABLE 3
*Liver protein regeneration properties*¹

CATEGORY OF INTEREST	D I E T F E D						
	Control	Fasting	Egg white	Lactal- bumin	Whole egg	Casein	Wheat gluten
Mg liver N per 100 gm body weight ²	122±1.2	91±1.6	105±1.1	125±1.8	109±1.8	117±1.6	107±1.4
Average body weight before fasting (gm)	228	211	222	223	221	217	222

¹ The authors are indebted to Mrs. Lois Barrows for her technical assistance in the assay.

² S.E. = Standard error.

nately superior to our whole egg or our egg white for the regeneration of liver proteins. This is not in complete accord with the findings of Harrison and Long ('45). Their data revealed no significant difference in the increase of liver nitrogen between rats fed with whole egg proteins and animals given casein or lactalbumin at a similar level. This discrepancy may be due to the difference in the samples used. It is also interesting to note that wheat gluten is not inferior to the egg proteins, according to this particular test.

Repletion with different proteins

In order to examine the plasma protein regeneration properties of the various proteins, the protein-depleted dogs were fed with diets containing one of the 5 different test proteins so that each animal received 0.35 gm of nitrogen and 80 cal. per kilogram body weight per day for a period of at least 4 weeks. The exception was that 0.6 gm of wheat gluten nitrogen was given instead of 0.35 gm, because of its low biological value. Determinations of total circulating plasma proteins, as well as albumin and globulins, were made before feeding and again two and 4 weeks after feeding (table 4). In summarizing our results we assumed the total circulating proteins, albumin or globulins immediately before feeding, to be 100%. Any change in plasma proteins due to protein feeding is expressed in terms of this figure.

Total circulating plasma protein

Our data (see table 4) demonstrate that supplementation of the protein-free diet with any one of the 5 proteins or two hydrolysates stimulated an increase in the total circulating plasma proteins, although the difference in the per cent increase over the depleted state was not great regardless of the nutritive values of the proteins fed. This fact in itself makes the quantitative determination of the increase in total circulating proteins not a very precise measure of the nutritive values of proteins. The lack of correlation is demonstrated by the fact that lactalbumin, with a nitrogen balance index of unity, did not promote the regeneration of plasma proteins as effectively as casein or the whole egg proteins having nitrogen balance indexes of 0.80 and 0.95, respectively. Egg white, which has the highest biological value among the 5 proteins, failed to bring about regeneration of plasma proteins to the same extent as casein even when feeding was continued for 8 weeks. A comparison between the whole proteins and their hydrolysates shows that the plasma protein regeneration property of casein was not improved by tryptic digestion,

TABLE 4
Effects of oral feeding of various proteins on total albumin and globulins of dogs depleted in proteins¹

PROTEIN FED	NO. DOGS USED	TOTAL CIRCULATING PROTEIN AFTER FEEDING FOR ²		TOTAL CIRCULATING ALBUMIN AFTER FEEDING FOR ²		TOTAL CIRCULATING GLOBULINS AFTER FEEDING FOR ²		A/G AFTER FEEDING FOR	
		2 wks.	4 wks.	2 wks.	4 wks.	2 wks.	4 wks.	0 wks.	2 wks. 4 wks.
Egg white	10	117 ± 5	132 ± 17	117 ± 8	133 ± 12	118 ± 5	126 ± 16	0.28	0.27 0.26
Lactalbumin	5	115 ± 10	138 ± 10	185 ± 21	252 ± 23	100 ± 9	100 ± 9	0.22	0.42 0.58
Whole egg	5	148 ± 7	154 ± 7	206 ± 21	289 ± 22	138 ± 6	129 ± 7	0.19	0.28 0.43
Casein	4	138 ± 9	141 ± 5	177 ± 17	216 ± 19	130 ± 8	124 ± 4	0.25	0.33 0.39
Wheat gluten	5	135 ± 4	135 ± 4	162 ± 6	168 ± 7	130 ± 5	129 ± 5	0.17	0.21 0.22
Lactalbumin hydrolysate	6	141 ± 8	145 ± 6	230 ± 5	362 ± 8	115 ± 6	98 ± 7	0.21	0.44 0.85
Casein hydrolysate	5	138 ± 7	150 ± 3	223 ± 4	238 ± 8	119 ± 11	124 ± 10	0.22	0.41 0.51

¹ All the proteins were given at a level of 0.35 gm N/kg body weight/day except wheat gluten, which was given at a 0.6 gm level.

² The total circulating proteins before feeding are taken as 100%. The figures after the ± signs are standard errors.

while the tryptic digest of lactalbumin was definitely superior in this respect to the whole protein. However, it should also be pointed out that casein is rapidly hydrolyzed by the proteolytic enzymes normally present in the digestive tracts of the animals, whereas lactalbumin is not.

Total circulating albumin

Since animals suffer a severe loss in the total circulating albumin as a result of depletion, it seems reasonable to expect that on repletion the albumin will be regenerated in preference to other plasma proteins. The data demonstrate that feeding each of these 5 proteins brought about a marked increase in albumin, ranging from 191% for lactalbumin to 117% for egg white after two weeks of feeding. The latter protein is surprisingly ineffective for the purpose of plasma albumin regeneration. It is interesting to note that statistically the rates of regeneration of albumin by the hydrolysates were significantly greater than those of the corresponding proteins (191% against 230% for lactalbumin and its hydrolysate; 177% against 223% for casein and its hydrolysate). After 4 weeks of feeding the regeneration of albumin continues except with wheat gluten, egg white, and casein hydrolysate.

Total circulating globulins

An examination of our data on globulin regeneration shows that protein supplementation brought about an increase in the total circulating globulins except with lactalbumin or its hydrolysate. Since protein depletion results in only a very slight decrease in the globulin content, it is to be expected that on repletion the increase of globulins is likewise less marked than that of the albumin. Our data do not demonstrate the existence of any significant difference in the regeneration of globulins among the various proteins. The most pertinent information gained in this study, therefore, is the lack of ability of lactalbumin or its hydrolysates to regenerate globulins, although they surpass other proteins in stimulating the

production of albumin. The results of electrophoretic analyses of various globulin fractions in the plasma of protein-depleted dogs before and after feeding the test proteins and hydrolysates are shown in table 5. It should be emphasized that the electrophoretic analyses of the individual globulin fractions are more subject to error than the albumin (Chow et al., '45; Petermann et al., '47). The data, nevertheless, demonstrate that the total circulating alpha globulins were slightly increased by feeding egg white proteins and wheat gluten, but

TABLE 5

Effects of oral feeding of different proteins on the various globulin fractions in the plasma of protein-depleted dogs

PROTEIN FED	NUMBER DOGS USED	TOTAL CIRCULATING GLOBULINS AFTER 4 WEEKS OF FEEDING ¹		
		alpha (1 and 2)	gamma	other globulins
Egg white	6	125 \pm 14	110 \pm 14	133 \pm 19
Lactalbumin	4	78 \pm 11	73 \pm 9	113 \pm 10
Whole egg	5	103 \pm 14	136 \pm 5	139 \pm 11
Casein	6	111 \pm 7	125 \pm 8	131 \pm 5
Wheat gluten	5	131 \pm 8	125 \pm 6	130 \pm 5
Lactalbumin hydrolysate	6	73 \pm 16	130 \pm 16	108 \pm 17
Casein hydrolysate	5	92 \pm 16	172 \pm 5	137 \pm 7

¹ The total circulating globulins before feeding are taken as 100%. The figures after the \pm signs are standard errors.

were decreased by feeding lactalbumin hydrolysate. Other proteins did not cause any significant change in this particular fraction. The gamma globulin, in which the antibodies lie, is also significantly increased by protein supplementation. Other globulin fractions (i.e., all other globulins excluding the alpha and gamma forms) were increased following the addition of the proteins to the protein-free diet except in the cases of lactalbumin and its hydrolysate.

The albumin to globulin ratios, as determined electrophoretically on samples of plasma taken immediately before

feeding and 4 weeks after feeding, are given in table 4. Our data demonstrate that this initial ratio varied between 0.17 and 0.28, with an average of 0.23. Repletion with the two hydrolysates or with lactalbumin brought the ratio to approximately normal.³ Casein and the whole egg proteins were less effective. The A/G values of the plasmas of the dogs receiving egg white and wheat gluten proteins were not increased.

DISCUSSION

The fact that various food proteins differ qualitatively in their ability to promote synthesis of plasma proteins has been established by investigators at Rochester (Holman et al., '34; Madden et al., '37, '38; McNaught et al., '36; Pommerenke et al., '35) Yale (Melnick et al., '36a, '36b, '37) and Columbia (Weech and Goettsch, '38a, '38b, '39). Table 6 was prepared in order to present a comparison of the data reported by these investigators with those we have obtained. The proteins are arranged according to the descending order of the nitrogen balance indexes, with egg white (index of 1.1) at the top and wheat gluten (index 0.3) at the bottom. According to the plasmapheresis technique, casein was definitely inferior to both lactalbumin and egg white. Determination with the Weech and Goettsch partial depletion technique ('38a), and on the basis of the increase of chemically estimated albumin showed that lactalbumin and egg white proteins were superior to casein and wheat gluten. In terms of the increase of total circulating proteins of the more severely depleted dogs, all 5 proteins were more or less alike. In terms of electrophoretic albumin, whole egg proteins and lactalbumin were superior to casein, wheat gluten and egg white. However, in terms of globulin regeneration lactalbumin or its hydrolysate was the most ineffective. In examining the results given in table 6 it is well to note that the methods of assay

³In our experience with more than 50 analyses involving 45 dogs which had been on a commercial dog food (Friskies, Albers Milling Company, Peoria, Ill.) and whole egg with occasional liver supplement for 4 to 6 weeks, the A/G ratio was 0.64 ± 0.026 .

were entirely different. As pointed out by Weech ('42), the plasmapheresis technique (Madden and Whipple, '40) measures *total capacity* for the formation of serum protein as opposed to the depletion technique (Weech) which measures relative differences in rate of the formation of albumin as estimated by the salt fractionation method. In the present study the animals were put on a protein-free diet for a period

TABLE 6

A comparison of the plasma protein regeneration properties of different proteins measured with various techniques

SOURCE OF PROTEIN	SERUM PROTEINS PRODUCED BY FEEDING 100 GM DIETARY PROTEIN DURING PLASMA- PHERESIS ¹	"CHEMICAL ALBUMIN" RISE IN THREE WEEKS (PARTIAL DEPLETION TECHNIQUE OF WEECH) ²	DEPLETION BY PROTEIN-FREE DIET FOR 6 TO 8 WEEKS: INCREASE IN TOTAL CIRCULATING ³		
			Protein	Albumin	Globulin
	gm	gm/100 ml	%	%	%
Egg white	17	0.616	130	160	130
Lactalbumin	18	0.773	130	260	100
Whole egg	.	0.602	150	290	130
Casein	10	0.425	140	220	120
Wheat gluten	..	0.207	140	170	130

¹ The figures refer to grams of serum proteins produced by feeding 100 gm of dietary protein.

² The figures refer to 0.15 plus the rise in albumin concentration (in gm/100 ml) in the serum during the week in which the food was fed. The constant, 0.15, is to be regarded as an allowance for maintenance.

³ The figures refer to increase in total circulating proteins expressed as per cent of the initial values before feeding, i.e., 100% for depleted animals.

of 6 to 8 weeks instead of three weeks (Weech's procedure), so that the plasma proteins reached a level of 4.0 to 4.5 gm %.

In addition to differences in the nutritional states of the dogs used, it must be mentioned that the 5 test proteins under comparison are not chemical entities. The nutritive quality of samples of lactalbumin might differ, depending on the process used in preparing them. In our experience, some commercial samples of lactalbumin gave nitrogen balance indexes ranging from 0.8 to 1.0. In the case of egg white, the situa-

tion is further complicated by the presence of the trypsin inhibitor (Harte, '45) and avidin, which might make some species of test animals deficient in biotin.

In the present paper are presented results of the measurement by different methods of the nutritive qualities of identical samples of 5 proteins. Hence, any variation in our results cannot be attributed to differences in the samples of proteins used. The results demonstrate that, in general, the commonly-used methods of measurement show a marked similarity in results except as regards liver protein regeneration and plasma protein regeneration properties. In the latter case, the criterion must specify whether the increase is of albumin or globulins.

The marked difference in the plasma protein regeneration properties of lactalbumin and casein is confirmed in whole proteins as in the hydrolysates, although the experiments were not designed to elucidate the explanation for this difference.

It is also of interest to note that there appears to be a significant difference between lactalbumin and its hydrolysate with respect to rate of albumin regeneration. It is conceivable that the hydrolysate is more efficiently utilized because of the availability of all the essential amino acids at one time. Digestion of lactalbumin by the animals not only takes time but may release different essential peptides at different rates (Melnick et al., '46). On the other hand, a protein like casein is readily hydrolyzed in the intestines and therefore it is reasonable to believe that it is absorbed as readily as its hydrolysate.

SUMMARY

The nutritive properties of 5 proteins (whole egg, egg white, lactalbumin, casein and wheat gluten) as well as two hydrolysates of casein and lactalbumin present in commonly consumed diets were measured with respect to their ability to support nitrogen balance, to promote growth, to replace body tissues, to regenerate liver proteins, and to stimulate the production of plasma proteins. It was found that, in general,

a parallelism exists among the results arrived at by the test methods used except for egg white, which is superior to the 4 other proteins according to the first three tests but not according to the last two.

Our data again demonstrate that lactalbumin or its hydrolysate favors the regeneration of albumin in protein, whereas other proteins will regenerate globulins as well. Examination of the A/G ratio after repletion demonstrates that the ratio returned to normal only after the feeding of lactalbumin, its hydrolysate, and casein hydrolysate.

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PTEROYLGLUTAMIC ACID BALANCE STUDIES ON MONKEYS¹

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TWO FIGURES

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The urinary excretion of pteroylglutamic acid (PGA) by normal human subjects receiving a mixed diet with no PGA supplement is quite low, being usually less than 5 μ g per 24 hours (Johnson, Hamilton and Mitchell, '45; Steinkamp, Shukers, Totter and Day, '46; Swendseid, Bird, Brown and Bethell, '47; Jukes, Franklin, Stokstad and Boehne, '47). Following the oral administration of 5 mg of PGA to such normal subjects, 30 to 50% of the test dose appears in the urine within 24 hours (Steinkamp et al., '46; Swendseid et al., '47; Jukes et al., '47). Most of the excreted material appears in the urine within the first 4 hours following dosage (Steinkamp et al., '46). The urinary excretion of a group of 7 hospital patients with various blood dyscrasias, before PGA treatment, did not differ significantly from that of normal subjects; however, following a test dose of PGA, the urinary returns were less than 11% of the dose (Steinkamp et al., '46).

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At least half of the administered oral dose of PGA is therefore unaccounted for. We might speculate that it could: (1) remain unabsorbed and appear in the feces; (2) be destroyed in the intestinal tract; (3) be absorbed and stored in the tissues; (4) be absorbed and destroyed in metabolism; (5) be absorbed and converted into some metabolite which does not stimulate the growth of *Streptococcus faecalis* or *Lactobacillus casei* and in that form be excreted in the urine; or (6) be disposed of by a combination of the foregoing. It seemed feasible to test the first of these possibilities by balance experiments upon a suitable primate. Such studies have been made upon the rhesus monkey and are reported in this paper.

EXPERIMENTAL

Young rhesus monkeys (2.1–2.9 kg) were housed in steel metabolism cages equipped with funnel-shaped stainless steel pans for the collection of urine. Routine hematological studies were made at weekly intervals. After a preliminary period of observation the animals were given the PGA-deficient diet described by Day and Totter ('47; see p. 317). This diet, if unsupplemented with PGA or some crude source of the vitamin, will eventually result in anemia, leukopenia, ulceration of the gums, diarrhea, and death. The anemia and leukopenia so induced respond dramatically to either pteroyltriglutamic acid (Day, Mims and Totter, '45) or to synthetic PGA (Totter, '46). Although the amount of this ration fed per monkey per day contains approximately 20 μ g of PGA, the diet consistently produces the deficiency syndrome. It may, in fact, be more suitable than highly refined diets for the production of PGA deficiency, since diets containing purified foodstuffs only have been shown to be deficient in another factor required by the monkey (Cooperman et al., '46).

In the experiments here described, daily urine and fecal collections were made for microbiological assay. The urine was collected under benzene, diluted to a convenient volume, and stored in a refrigerator. The feces were collected,

weighed and mixed, and a weighed sample of them was homogenized in a Waring Blendor and stored in the refrigerator. The microbiological assays (Mitchell and Snell, '41) employed *Streptococcus faecalis* (American Type Culture Collection no. 8043), using synthetic PGA as a standard. Turbidimetric readings were made with a Coleman spectrophotometer. In one series of assays, to be referred to later, simultaneous determinations were also made using *Lactobacillus casei*. The urines were assayed for free PGA only. Assays were made on the feces for both free and conjugated PGA; for the conjugated PGA, conjugase preparations from rat liver (Mims, Totter and Day, '44), chicken pancreas (Laskowski et al., '45) and hog kidney (Bird et al., '45) were employed. The highest accurate value obtained with the three enzymes was used. The period of collection for each experiment was three weeks or longer.

In the balance experiments here reported, the following materials were fed to monkeys, as daily supplements to the deficient diet, under suitably controlled conditions: 100 μ g of synthetic PGA; 1000 μ g of synthetic PGA; 100 μ g of pterotic acid; 2.5 gm of yeast extract,² which contained approximately 100 μ g of PGA, largely as the conjugate. Balance studies were also made on a control monkey receiving no supplement to the deficient diet. The 100 μ g level was chosen as a daily supplement because this appears to be approximately the minimum protective dose of PGA for a young monkey weighing 2-3 kg. Although, on the basis of microbiological assays of materials previously shown to protect the monkey, Day and Totter ('47) tentatively set the minimum daily protective level at 40 to 80 μ g PGA as supplement to the deficient diet, subsequent unpublished experiments with synthetic PGA have indicated that 100 μ g is probably the minimum daily dose necessary to protect against deficiency manifestations. The monkey which was given 2.5 gm of yeast extract likewise received the minimum protective amount of PGA, in the form of the conjugate equivalent to about 100 μ g of PGA.

² Difco.

Although under certain circumstances the monkey may not satisfactorily utilize the conjugate, an amount of conjugate equivalent to 100 μ g of PGA was found completely protective when fed in the form of yeast in long-term experiments (Day and Totter, '47, pp. 319-320).

The data obtained were treated statistically by the analysis of variance method (Snedecor, '46).

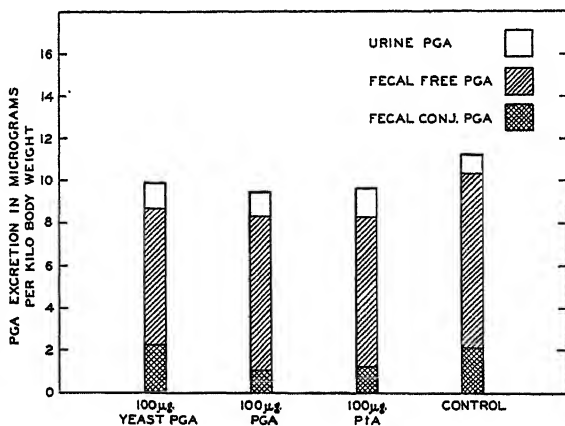


Fig. 1 Average daily excretion data, expressed in terms of microbiologically equivalent amounts of PGA, on monkeys receiving daily the amounts of the materials indicated below the respective bars.

RESULTS AND DISCUSSION

The monkeys receiving 100 μ g of PGA, 1000 μ g of PGA, and 2.5 gm of yeast extract maintained normal blood pictures throughout the studies, as would be expected. During the short period of the balance study, the monkey receiving 100 μ g of pterioic acid was also free of deficiency manifestations; although we assume that pterioic acid—at this level at least—would not protect the monkey, the experiment was not continued long enough to prove this point.

Figure 1 presents in graphic form the average daily excretions of PGA (i.e., substances stimulating the growth of *Streptococcus faecalis*) by monkeys fed 2.5 gm of yeast³ (con-

³ See footnote 2, p. 805.

taining conjugate equivalent to 100 μ g of PGA), 100 μ g of synthetic PGA, and 100 μ g of synthetic pterioic acid (PtA); and by a control monkey receiving no supplement to the deficient diet. In this figure the excretion data are plotted per kilogram of body weight. The control monkey was somewhat larger (2.96 kg) than the other animals (2.08–2.43 kg). When expressed in terms of total outputs, the deficient control excreted significantly greater amounts than the other monkeys. It therefore seemed more valid to make comparisons per unit of body weight. Expressed in this way, as is shown in the figure, there appear to be no significant differences among the 4 animals as regards urinary excretion, fecal excretion, and total excretion. A statistical study of the data has confirmed the impression that the differences are not significant.

Several conclusions seem immediately apparent and fully justified on the basis of this experiment. The minimum daily protective dose of synthetic PGA (100 μ g), when given by mouth, does not significantly increase either the urinary or the fecal excretion of microbiologically active substances. It is thus obvious that at this level of intake the PGA does not pass through the intestinal tract unchanged; from these data we cannot conclude whether it is (1) altered or destroyed in the intestinal tract or (2) absorbed from the tract.

Pterioic acid, when fed at the 100 μ g per day level, did not appreciably increase the excretion of active substances in either the urine or the feces. Here again, we cannot decide on the basis of these data whether the pterioic acid was chemically altered in the tract and excreted in the feces in a form microbiologically inactive, or whether it was absorbed from the tract. In any event, it did not appear in the urine in measurable quantities, for the urinary level of microbiologically active material was not increased. Furthermore, urine samples from this monkey and from the monkey receiving 100 μ g of PGA were assayed with both *Lactobacillus casei* and *Streptococcus faecalis*. The assay values obtained with the two organisms were in substantial agreement for

both animals. This constitutes additional evidence that orally administered pterioic acid, at this level of intake, does not appear in the urine in measurable quantities.

The conclusions to be drawn from the yeast experiment may be no less significant. The conjugate supplied by 2.5 gm of yeast extract did not alter the total fecal output or the conjugate fecal output to any important extent. Actually the data (fig. 1) show a fecal conjugate excretion by the yeast monkey twice that of the PGA monkey. It must be remembered, however, that the measurement of the conjugate, even in the hands of a skilled technician, is subject to several cumulative errors with the methods now available. Because of these possible errors and the small quantity of conjugate in the fecal specimens, we attach no great significance to these apparent differences in conjugate content. Whether such differences are significant or not, this point stands out clearly: the average daily conjugate content of the feces of the monkey receiving yeast extract was only 5% of the yeast PGA conjugate fed. Quite evidently, crude yeast extract conjugate does not pass through the intestinal tract of the monkey unchanged.

A general conclusion seems warranted from this experiment. The young rhesus monkey on a PGA-deficient diet continues to excrete a limited amount of material having microbiological activity like that of PGA. In this experiment the daily output in the urine averaged about 1 μ g, and in the feces about 10 μ g, per kilogram of body weight. These excretion levels must be looked upon as basal levels. They were not increased by an oral intake of synthetic PGA sufficient to supply the minimum requirement of the monkey (100 μ g per monkey per day), nor by 100 μ g of pterioic acid, nor by yeast conjugate equivalent to 100 μ g of PGA.

This concept of a continuous basal urinary and fecal excretion of PGA by the monkey must not be interpreted as implying a rigidly *constant* excretion. Such is not the case. Repeated observations of a given animal receiving the same diet for a long period have shown significant alterations in

output from time to time. Whether these changes are the result of seasonal or temperature changes, minor differences in diet constituents from batch to batch, or to other factors, we cannot say at this time. These findings do emphasize the importance of rigid control in such balance experiments, either by the use of simultaneous experiments or by consecutive experiments on a single animal.

Figure 2 represents the data from an experiment in which the intakes were 1000 and 100 μg of synthetic PGA respec-

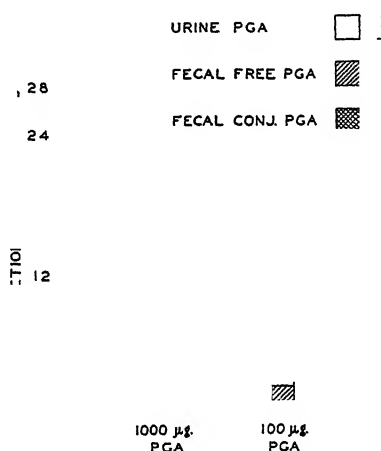


Fig. 2 Average daily excretion data, expressed in terms of microbiologically equivalent amounts of PGA, on a monkey receiving 1000 μg and 100 μg of synthetic PGA daily, in successive experimental periods.

tively, the former value a 10-fold increase over the minimum daily requirement of the monkey. These were successive studies on the same animal, and since the monkey did not change weight during the series, comparisons are valid without taking body weight into consideration. It is immediately apparent from an inspection of the figure that the outputs in both urine and feces were significantly greater during the 1000 μg period than during the 100 μg period. Statistical treatment of the data confirms the significance of these differences. Viewing our findings superficially, we would con-

clude that a 10-fold increase in intake resulted in a three-fold increase in output. Examining the data in the light of the experiment summarized in figure 1 and similar unpublished experiments, however, we may properly conclude that the output of the animal during the 100 μ g intake period was the "basal" output, which would have been essentially the same had no PGA been added to the deficient diet. If we subtract this "basal" urinary output (4.8 μ g) from the average urinary output during the 1000 μ g period, we find that the increase in urinary output was approximately 10 μ g, which is only about 1% of the daily dose. One might speculate that this low urinary return was the result of low tissue levels of PGA, in which case the feeding of large daily doses over a long period should result in tissue saturation and an increase in urinary output. However, no such increase has been observed in a monkey which has received 1 mg of PGA daily for 80 days. This low percentage of urinary excretion is in sharp contrast to the response of the normal adult human, who excretes in the urine 30–50% of a 5 mg oral test dose of PGA within 24 hours. This difference may be related to the peculiar susceptibility of the young rhesus monkey to PGA deficiency.

It is also clear from this experiment that orally administered synthetic PGA does not, to any important extent, pass unchanged through the intestinal tract of the monkey; although the higher level of feeding resulted in an increased fecal output, that daily fecal increase was less than 1% of the daily dose.

The fate of the other 98% of the 1000 μ g dose of PGA is yet to be elucidated. We cannot conclude from these experiments whether it is altered or destroyed in the intestinal tract, whether it is absorbed and stored in the tissues, or whether it is absorbed and then altered or destroyed in metabolism.

SUMMARY

Young rhesus monkeys were given a diet known to produce the anemia, leukopenia, and other manifestations of pteroyl-

glutamic acid (PGA) deficiency. Daily urine and fecal collections were made and these excreta assayed for PGA, using *Streptococcus faecalis*. Suitable conjugase preparations were employed in the fecal analyses. Each experimental period lasted 21 days or longer.

The following daily additions were made to the diets of such animals, under suitably controlled conditions: 100 μ g of synthetic PGA (which is the approximate minimum daily requirement); 100 μ g of synthetic pterioic acid; 2.5 gm of yeast extract, containing approximately 100 μ g of PGA, largely in the form of the conjugate. The average daily urinary and fecal PGA excretions of these monkeys, when expressed on a body weight basis, were not significantly different from the excretions of a control monkey receiving the deficient diet only. In this series the average daily urinary outputs were approximately 1 μ g, and the average fecal outputs were between 8 and 10 μ g, expressed in terms of synthetic PGA per kilogram of body weight. These may be considered "basal" levels of excretion.

It is concluded that neither synthetic PGA, synthetic pterioic acid, nor crude yeast PGA conjugate, when fed at the 100 μ g level, pass through the intestinal tract of the young rhesus monkey unchanged.

Increasing the daily intake of synthetic PGA to 1000 μ g resulted in a three-fold increase in urinary and fecal output over the "basal" level. Notwithstanding, the actual urinary increase was only about 1% of the oral dose and the fecal increase much less than 1% of the dose. Obviously, even at high levels of intake, synthetic PGA does not, to any significant extent, pass through the intestinal tract of the monkey unchanged.

The low percentage urinary return of large daily oral doses of synthetic PGA by the monkey is in sharp contrast to the response of the normal adult human, who excretes 30-50% of a 5 mg oral dose within 24 hours.

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THE ROLE OF THE TIME FACTOR IN FEEDING SUPPLEMENTARY PROTEINS

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It has been demonstrated in previous experiments (Geiger, '47; Cannon et al., '47; Schaeffer and Geiger, '47), that incomplete amino acid mixtures cannot be stored in the animal organism, and that protein synthesis occurs only when all the essential building stones are available simultaneously. These results were recently confirmed by Harte, Travers and Sarich ('48), who demonstrated that alternate feeding of wheat gluten and a protein hydrolysate resulted in significantly poorer growth than that obtained when both sources of nitrogen were fed together. These results are further supported by the experiments of Yeshoda and Damodaran ('47), who found that injections of tryptophane into animals on tryptophane-free diets had a hemopoietic effect but failed to prevent the loss of body weight. Finally, Pearce, Sauberlich and Baumann ('47) have shown for mice, and Sauberlich and Baumann ('48) for rats, that urinary amino acid losses are increased when the animals are fed incomplete amino acid mixtures. In all these experiments, only amino acids themselves or amino acid mixtures were investigated. The results, therefore, are primarily of theoretical interest and their practical value is restricted to those instances when mixtures of the amino acids are fed or injected. The proteins actually present

in food differ from the amino acids in that they are slowly digested and absorbed and therefore it seemed advisable to ascertain whether the findings discussed above could be extended to the dietary proteins; hence the present investigation of the metabolic fate of the proteins which lack one or more essential building stone and therefore do not promote growth satisfactorily.

In order to study this problem it was decided to select pairs of proteins which, fed separately, do not promote growth, but which, when given together, supplement each other's amino acid deficiencies. By alternately feeding such protein pairs the author intended to duplicate the conditions which obtained in earlier experiments with delayed supplementation of amino acids.

METHODS

The composition of the basal diet was as follows: Corn starch, 3050 gm (78.3%); rice bran concentrate, 400 gm (10.26%); cottonseed oil, 200 gm (5.13%); U.S.P. salt mixture, 200 gm (5.13%); fish oil (1 gm contains 2,000 I.U. vitamin A and 400 I.U. vitamin D) 50 gm (1.27%); riboflavin, 75 mg; Ca pantothenate, 150 mg; choline chloride, 2.5 gm. The protein sources used and their respective nitrogen contents in per cent were as follows: cultured yeast (Strain G),¹ N (Kjeldahl) 6.9; wheat gluten,² N (Kjeldahl) 12.14; blood protein,³ N (Kjeldahl) 13.43.

Male Sprague-Dawley rats weighing from 45 to 50 gm were used. The animals were placed in individual cages. Body weight and food consumption were determined daily. A detailed description of the technic of feeding has been given in an earlier paper (Geiger, '47).

The rats of the different groups received food from 8:00 A.M. to 6:00 P.M. and from 8:00 P.M. to 6:00 A.M. The feeding cups were removed for two-hour intervals between the feeding periods.

¹ Anheuser Busch.

² Pure Gluten Food Co.

³ Viobin Corp.

EXPERIMENTAL

The results of these experiments are presented in table 1.

TABLE 1

Growth of rats during 16 days on feeding of supplementary pairs of proteins

PLAN OF FEEDING	PAIRS OF SUPPLEMENTARY PROTEINS		
	Wheat gluten + blood protein	Yeast + blood protein	Wheat gluten + yeast
	gm	gm	gm
Fed together	Group III	Group III	Group III
	19	16	24
	22	15	22
	18	17	28
	30	22	30
Fed separately	Group IV	Group IV	Group IV
	3	—9	—4
	2	—3	3
	—2	—5	4
	—4	2	6

Experiments with wheat gluten and blood protein

The first pair of supplementary proteins studied consisted of wheat gluten and blood protein. Wheat gluten is deficient in lysine (Mitchell and Block, '46), while blood protein is especially low in isoleucine (Albanese, '45).

Four groups of male rats were used, each consisting of 4 animals of practically equal weight (45–50 gm). Group I received a diet containing 9% blood protein, group II one containing 9% wheat gluten, and group III a ration containing 4.5% blood protein and 4.5% wheat gluten; group IV was fed a diet containing 9% blood protein for 10 hours and, for the next 10-hour feeding period, one containing 9% wheat gluten.

The animals of groups I and II showed no growth for a period of 16 days, indicating that neither blood protein nor wheat gluten by itself promotes growth when fed at a 9% level. During the 16-day period the animals of group III

gained 19, 18, 22, and 30 gm, indicating that blood protein and wheat gluten fed together promote growth, i.e., that they supplement each other's amino acid deficiencies. Groups III and IV were subjected to paired feeding; their total food and protein intakes were practically the same, being 130 ± 5 gm total food intake per animal for 16 days. In spite of this, the animals of group IV which were fed rotating diets containing blood protein and wheat gluten showed no appreciable growth. One animal gained 3 gm; the second, 2 gm; the third and 4th animals lost 2 and 4 gm, respectively.

These experiments indicate that the two proteins under examination exert their complementary influences only when fed simultaneously.

Experiments with yeast and blood protein

In the second group of experiments yeast was used as a protein complementary to the blood protein. The method of feeding was the same as that used in the preceding experiments with wheat gluten.

The animals of group I received 9% blood protein and of group II 9% yeast protein in their diets. Neither of these groups showed increase in growth. The animals of group III, receiving a diet containing 4.5% blood and 4.5% yeast protein, gained 16, 15, 17 and 22 gm. In group IV, where diets containing 9% yeast and 9% blood protein were fed in rotation at 10-hour intervals for 16 days, no growth occurred. The changes in weight were -9, -3, +2 and -5 gm for the 4 groups, respectively. The paired-fed rats of groups III and IV consumed 145 ± 12 gm of food each for the 16-day period.

These experiments indicate that yeast and blood proteins supplement each other's growth-promoting properties only when fed simultaneously.

Experiments with yeast and wheat gluten

In a third group of experiments we selected, as complementary proteins, yeast and wheat gluten. According to the

data of Block and Bolling ('45), yeast contains 6% lysine while wheat gluten contains only 1.1%. Methionine, on the other hand, is present in high concentration in wheat gluten (5.5%), and is low in yeast (2%). In control experiments it was shown in confirmation of the data of Klose and Fevold ('45), that yeast protein fed on a 9% basis does not promote growth in infant rats; wheat gluten was likewise unable to promote growth when fed on this basis.

In order to investigate their complementary effects on each other, a diet was fed containing 4.5% wheat gluten and 4.5% yeast protein to 4 rats in group III. These animals showed a very good growth, gaining 24, 22, 28 and 30 gm, respectively, during the 16-day experimental period. To group IV was fed alternately, for two 10-hour periods, a diet containing 9% wheat gluten, and one containing 9% yeast protein. The change in body weight was insignificant; one animal lost 4 gm and the others gained 3, 4 and 6 gm, respectively. The total food consumption for each animal subjected to paired feeding was 222 ± 8 gm.

These experiments indicate that when yeast protein and wheat gluten protein are fed together they supplement each other's amino acid deficiencies but that feeding these proteins at separate times interferes with their effective complementary actions.

It may therefore be concluded that, as in our earlier experiments with amino acid mixtures, the building stones of deficient proteins cannot be stored, and that the delayed provision of the missing factors is ineffective not only when they are fed as free amino acids but also when they are supplied in the form of proteins.

A consequence of these experiments seems to be the conclusion that supplementary proteins of individually low biological value must be fed together in order to achieve their optimum utilization.⁴

After presenting these results at a meeting of the American Association for the Advancement of Science, San Francisco,

⁴ Editorial, *Nutrition Reviews*, 5: 316 (1947).

1948, our attention was called to a paper by Henry and Kon ('46), who found that milk and potato, and bread and cheese, exhibit supplementary relationships only if fed together and not if fed separately on alternate days. These results are in complete agreement with our conclusions. It is, however, possible that in the experiments of Henry and Kon, where only highly complex foods such as bread, cheese or potatoes were investigated, some factors other than protein may be at least partially responsible for the results.

SUMMARY

The question was investigated of whether supplementary proteins of low biological value promote the growth of infantile rats when fed individually at different times.

It was found that yeast, blood and wheat gluten proteins do not promote growth when fed individually at a 9% level. Diets containing wheat gluten + blood protein, or yeast + blood protein, or yeast + wheat gluten protein, have satisfactory growth-promoting properties. If, however, these same pairs of proteins are fed separately with lapses of time between feedings, they do not supplement each other, as is indicated by the lack of resulting growth.

These experiments show that delayed provision of the missing essential amino acids is ineffective, not only when fed as the amino acids themselves (Geiger, '47), but also when they are supplied in the form of proteins.

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BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1949 Award of \$1000, and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the judges may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1949. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 15, 1949. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

JAMES M. ORTEN
*College of Medicine,
Wayne University,
Detroit, Michigan*

CHAIRMAN, NOMINATING COMMITTEE

MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1949 Award of \$1000, established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the spring of 1949.

The Award will be given to the laboratory (non-clinical) or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year, January 1 to December 31, the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1948 must be in the hands of the Chairman of the Nominating Committee by January 15, 1949. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

HAROLD H. WILLIAMS
Cornell University, Ithaca, N. Y.

CHAIRMAN, NOMINATING COMMITTEE

OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of \$1000, established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1949 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 15, 1949.

D. W. WOOLLEY

*Rockefeller Institute for
Medical Research, New York, N. Y.*

CHAIRMAN, NOMINATING COMMITTEE

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